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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/786,214	06/14/2001	Michael Probst-Kepper	L0461/7102	6577

7590 12/07/2005

John R Van Amsterdam
600 Atlantic Avenue
Boston, MA 02210

EXAMINER	
DIBRINO, MARIANNE NMN	

ART UNIT	PAPER NUMBER
1644	

DATE MAILED: 12/07/2005

Please find below and/or attached an Office communication concerning this application or proceeding.

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09/786,214	06/14/2001	Michael Probst-Kepper	L0461/7102	6577

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John R Van Amsterdam
600 Atlantic Avenue
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EXAMINER	
DIBRINO, MARIANNE NMN	

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1644	

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Office Action Summary	09/786,214	PROBST-KEPPER ET AL.
	Examiner: DiBrino Marianne	Art Unit: 1644

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *01 December 2004*.

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-3,7,58,59 and 65-71* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1,2,7,58,59 and 66-70* is/are rejected.

7)☐ Claim(s) *3,65 and 71* is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☒ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ Other: _____.

DETAILED ACTION

1. Applicant's amendment filed 12/1/04 is acknowledged and has been entered.

2. Applicant is reminded of Applicant's election of Group I (claims 1-3, 7, 8, 58 and 59), and species of polypeptide comprising SEQ ID NO: 5 in Applicant's response filed 2/6/04.

Claims 1-3, 7, 58, 59 and 65-71 are currently being examined.

3. The oath or declaration is defective. A new oath or declaration in compliance with 37 CFR 1.67(a) identifying this application by application number and filing date is required. See MPEP §§ 602.01 and 602.02.

The oath or declaration is defective because: The first line of the specification claims priority to Application serial no. 60/099,077 filed September 4, 1998 that is not listed in the declaration.

The Examiner acknowledges Applicant's statement in the amendment filed 12/1/04 that an application data sheet was filed, however, the said application data sheet does not appear to have been filed.

The following are new grounds of rejection necessitated by Applicant's amendment filed 12/1/04.

4. The following is a quotation of the first paragraph of 35 U.S.C. 112:

The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

5. Claims 1, 2, 7, 58, 59 and 66-70 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

The specification does not provide adequate written description of the claimed invention. The legal standard for sufficiency of a patent's (or a specification's) written description is whether that description "reasonably conveys to the artisan that the inventor had possession at that time of the. . .claimed subject matter", Vas-Cath, Inc. V. Mahurkar, 19 USPQ2d 1111 (Fed. Cir. 1991). In the instant case, the specification does not convey to the artisan that the Applicant had possession at the time of invention of the claimed: (1) isolated polypeptide/composition thereof comprising the amino acid sequence of a "functional amino acid substitution variant" of SEQ ID NO: 12 "that retains immunogenicity", (2) or a fragment having, i.e., comprising, at least 14 consecutive amino acid residues of SEQ ID NO: 5, (3) a composition comprising an

immunogenic fragment of SEQ ID NO: 5, or which said composition comprises an immunogenic fragment, said immunogenic fragment comprises the amino acid sequence of SEQ ID NO: 12, (4) an isolated immunogenic polypeptide comprising the amino acid sequence of SEQ ID NO: 5, SEQ ID NO: 9 or SEQ ID NO: 12.

The instant claims encompass: (1) an isolated polypeptide/composition thereof comprising the amino acid sequence of a "functional amino acid substitution variant" of SEQ ID NO: 12 "that retains immunogenicity", i.e., it can stimulate an antibody response or it can stimulate a T cell response to a different HLA/T cell combination than SEQ ID NO: 12, (2) a fragment having, i.e., comprising, at least 14 consecutive amino acid residues of SEQ ID NO: 5 that does not contain SEQ ID NO: 12 and does not stimulate the same HLA/CTL combination, (3) a composition comprising an immunogenic fragment of SEQ ID NO: 5 that does not contain SEQ ID NO: 12 and does not stimulate the same HLA/CTL combination, or which said composition comprises an immunogenic fragment, said immunogenic fragment comprises the amino acid sequence of SEQ ID NO: 12 but does not further comprise flanking amino acid residues present in SEQ ID NO: 5, (4) an isolated immunogenic polypeptide comprising the amino acid sequence of SEQ ID NO: 5, SEQ ID NO: 9 or SEQ ID NO: 12 that does not further comprise flanking amino acid residues present in the alt.M-CSF sequence, i.e., that is not a subsequence of alt.M-CSF tumor rejection protein. In addition, the said peptide/fragment composition, thereof can comprise amino acid residues that flank the said sequences in the peptide or protein of origin, or can be any number of undisclosed and unrelated sequences or can be non-peptidic in nature. There is insufficient disclosure in the specification on the said peptide/fragment/composition including vaccine thereof.

The specification discloses that expression of alt.M-CSF is detected in normal hepatocytes and not exclusively in [renal] tumor cells (page 42 at lines 16-20). The specification further discloses that the peptide SEQ ID NO: 12 is derived from the translation of an alternative open reading frame of the normal human M-CSF cDNA and is recognized by a CTL line from a renal carcinoma patient as well as on two allogeneic HLA-B*3501 positive renal cell carcinoma lines (page 40 at lines 13-16). The specification further discloses that N- or C-terminal truncations of SEQ ID NO: 12 failed to sensitize allogenic HLA-B*3501 positive EBV-B cells, SEQ ID NO: 12 appearing to be the minimal peptide (page 40 at lines 4-12).

The specification discloses that functional a variant of an alt.M-CSF immunogenic polypeptide is a molecule which contains one or more modifications to the primary amino acid sequence of an alt.M-CSF immunogenic polypeptide and retains the HLA class I binding properties disclosed as well as the ability to stimulate proliferation and/or activation of $CD8^+$ T lymphocytes (page 11 at lines 10-14). The specification discloses that modifications to create a functional variant include enhancing a property such as peptide stability in an expression system, more stable peptide/HLA binding, or providing a novel activity or property such as the addition of an antigenic epitope or a detectable moiety, or providing a different amino acid sequence that produces the same or similar T cell stimulatory properties (page 11 at lines 14-20). The specification discloses that the amino acid sequences of alt.M-CSF immunogenic

polypeptides may be of natural or non-natural origin, that is they may comprise a natural alt.M-CSF immunogenic polypeptide molecule or they may comprise a modified sequence as long as the amino acid sequence retains the ability to stimulate CTL when presented and binds to an HLA Class I molecule such as HLA-B*3501 (page 11 at lines 28-32).

The specification discloses that methods for identifying functional variants of an alt.M-CSF immunogenic polypeptide include selecting an alt.M-CSF polypeptide, an HLA molecule that binds the said polypeptide, and a T cell that is stimulated by the said polypeptide or a fragment thereof, adding, deleting or substituting a first or second amino acid residue and testing for binding to HLA and/or stimulation of a T cell (paragraph spanning pages 6 and 7).

The specification discloses other methods for identifying functional variants of the alt.M-CSF immunogenic polypeptides rely upon the development of amino acid sequence motifs to which potential epitopes may be compared or that motif analysis may be used in design of such polypeptides, experimental ranking schemes may be used and the stimulation of the T cell are determined according to standard procedures (page 13 at lines 13-32, page 14 at lines 1-22 and page 15 at lines 3-12).

The specification discloses methods for identifying a candidate mimetic of an alt.M-CSF polypeptide that is not necessarily a peptide, but is a functional variant (page 8 at lines 1-12). The specification discloses that exemplary polypeptides are processed translation products of SEQ ID NO: 4 and that the said polypeptides may be any length as long as they are processed to a final form that encompasses SEQ ID NO: 12. The specification discloses that SEQ ID NO: 12 may have added amino acid residues that correspond to the alt.M-CSF polypeptide of SEQ ID NO: 5, or may be unrelated (page 10 at lines 11-24).

In claims involving chemical materials, generic formulae usually indicate with specificity what the generic claims encompass. One skilled in the art can distinguish such a formula from others and can identify many of the species that the claims encompass. Accordingly, such a formula is normally an adequate description of the claimed genus. However, a generic statement such as "functional amino acid substitution variant that retains immunogenicity" or "a fragment thereof having at least 14 consecutive amino acid residues of SEQ ID NO: 5" or "composition comprising an immunogenic fragment of SEQ ID NO: 5" does not describe the claimed peptide/fragment/composition thereof, except by the property of retaining some form of immunogenicity, or containing at least 14 consecutive amino acid residues of SEQ ID NO: 5 which may not include SEQ ID NO: 12, or that comprises any immunogenic fragment of SEQ ID NO: 5 that may not include SEQ ID NO: 12 that stimulates a humoral or cellular immune response. It does not specifically define any of the peptides/functional amino acid substitution variants/immunogenic fragments that fall within its definition. It does not define any structural features commonly possessed by members of the genus that distinguish them from others, other than the variant has to bind some HLA molecule and stimulate some T cell or some antibody response. One skilled in the art therefore cannot, as one can do with a fully described genus, visualize or recognize the identity of the members of the genus. In addition, a definition by

function does not suffice to define the genus because it is only an indication of what the property the peptide has, rather than what it is. See Fiers, 984 F.2d at 1169-71, 25 USPQ2d at 1605-06. It is only a definition of a useful result rather than a definition of what achieves that result. Many such species may achieve that result. The description requirement of the patent statute requires a description of an invention, not an indication of a result that one might achieve if one made that invention. See In re Wilder, 736 F.2d 1516, 1521, 222 USPQ 369, 372-73 (Fed. Cir. 1984) (affirming rejection because the specification does "little more than outlin [e] goals appellants hope the claimed invention achieves and the problems the invention will hopefully ameliorate.") Accordingly, naming a type of material generally known to exist, in the absence of knowledge as to what that material consists of, is not a description of that material.

One of ordinary skill in the art would reasonably conclude that the disclosure fails to provide a representative number of species to describe the genus as broadly claimed.

Applicant's arguments in the amendment filed 12/1/04 have been fully considered but are not persuasive.

Applicant's arguments are of record in the said amendment on pages 5-7.

It is the Examiner's position that claim 1 recites an isolated polypeptide comprising the amino acid sequence of SEQ ID NO: 5, or a fragment thereof having at least 14 consecutive amino acids of SEQ ID NO: 5, i.e., the polypeptide comprises the fragment, and so the sequence of the said isolated polypeptide can be of any length or composition flanking either end of the said fragment. It is the Examiner's further position with regard to Applicant's argument to an isolated immunogenic polypeptide comprising the amino acid sequence of SEQ ID NO: 5 or SEQ ID NO: 12, that the amino acid residues flanking the polypeptide can be any amino acid residues up to any length.

6. Claims 1, 2, 7, 58, 59 and 66-70 are rejected under 35 U.S.C. 112, first paragraph, because the specification, while being enabling for making and/or using a peptide consisting of the sequence of SEQ ID NO: 5, 9 or 12, does not reasonably provide enablement for making and/or using an isolated polypeptide/composition thereof comprising the amino acid sequence of (1) a "functional amino acid substitution variant" of SEQ ID NO: 12 "that retains immunogenicity", i.e., it can stimulate an antibody response or it can stimulate a T cell response to a different HLA/CTL cell combination than SEQ ID NO: 12, (2) a fragment having, i.e., comprising, at least 14 consecutive amino acid residues of SEQ ID NO: 5 that does not contain SEQ ID NO: 12 and does not stimulate the same HLA/CTL combination, (3) a composition comprising an immunogenic fragment of SEQ ID NO: 5 that does not contain SEQ ID NO: 12 and does not stimulate the same HLA/CTL combination, or which said composition comprises an immunogenic fragment, said immunogenic fragment comprises the amino acid sequence of SEQ ID NO: 12 but does not further comprise flanking amino acid residues present in SEQ ID NO: 5, (4) an isolated immunogenic polypeptide comprising the

amino acid sequence of SEQ ID NO: 5, SEQ ID NO: 9 or SEQ ID NO: 12 that does not further comprise flanking amino acid residues present in the alt.M-CSF sequence, i.e., that is not a subsequence of alt.M-CSF tumor rejection protein. The state of the art is such that it is unpredictable in the absence of appropriate evidence whether the claimed invention can be made and or used. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make or use the invention commensurate in scope with these claims.

The specification discloses that expression of alt.M-CSF is detected in normal hepatocytes and not exclusively in [renal] tumor cells (page 42 at lines 16-20). The specification further discloses that the peptide SEQ ID NO: 12 is derived from the translation of an alternative open reading frame of the normal human M-CSF cDNA and is recognized by a CTL line from a renal carcinoma patient as well as on two allogeneic HLA-B*3501 positive renal cell carcinoma lines (page 40 at lines 13-16). The specification further discloses that N- or C-terminal truncations of SEQ ID NO: 12 failed to sensitize allogenic HLA-B*3501 positive EBV-B cells, SEQ ID NO: 12 appearing to be the minimal peptide (page 40 at lines 4-12).

The specification discloses that functional a variant of an alt.M-CSF immunogenic polypeptide is a molecule which contains one or more modifications to the primary amino acid sequence of an alt.M-CSF immunogenic polypeptide and retains the HLA class I binding properties disclosed as well as the ability to stimulate proliferation and/or activation of $CD8^+$ T lymphocytes (page 11 at lines 10-14). The specification discloses that modifications to create a functional variant include enhancing a property such as peptide stability in an expression system, more stable peptide/HLA binding, or providing a novel activity or property such as the addition of an antigenic epitope or a detectable moiety, or providing a different amino acid sequence that produces the same or similar T cell stimulatory properties (page 11 at lines 14-20). The specification discloses that the amino acid sequences of alt.M-CSF immunogenic polypeptides may be of natural or non-natural origin, that is they may comprise a natural alt.M-CSF immunogenic polypeptide molecule or they may comprise a modified sequence as long as the amino acid sequence retains the ability to stimulate CTL when presented and binds to an HLA Class I molecule such as HLA-B*3501 (page 11 at lines 28-32).

The specification discloses that methods for identifying functional variants of an alt.M-CSF immunogenic polypeptide include selecting an alt.M-CSF polypeptide, an HLA molecule that binds the said polypeptide, and a T cell that is stimulated by the said polypeptide or a fragment thereof, adding, deleting or substituting a first or second amino acid residue and testing for binding to HLA and/or stimulation of a T cell (paragraph spanning pages 6 and 7).

The specification discloses other methods for identifying functional variants of the alt.M-CSF immunogenic polypeptides rely upon the development of amino acid sequence motifs to which potential epitopes may be compared or that motif analysis may be used in design of such polypeptides, experimental ranking schemes may be used and the stimulation of the T cell are

determined according to standard procedures (page 13 at lines 13-32, page 14 at lines 1-22 and page 15 at lines 3-12).

The specification discloses methods for identifying a candidate mimetic of an alt.M-CSF polypeptide that is not necessarily a peptide, but is a functional variant (page 8 at lines 1-12). The specification discloses that exemplary polypeptides are processed translation products of SEQ ID NO: 4 and that the said polypeptides may be any length as long as they are processed to a final form that encompasses SEQ ID NO: 12. The specification discloses that SEQ ID NO: 12 may have added amino acid residues that correspond to the alt.M-CSF polypeptide of SEQ ID NO: 5, or may be unrelated (page 10 at lines 11-24).

The specification discloses that alt.M-CSF immunogenic polypeptides such as SEQ ID NO: 12 which are presented by MHC and recognized by CTL can be combined with peptides from other tumor rejection antigens to form polytopes, in order to make composite polypeptides that correspond to the different combination of epitopes representing a subset of tumor rejection antigens expressed in a particular patient or expressed by a tumor type, and the said polytopes administered to induce or enhance an immune response. However, the specification does not disclose use of compositions comprising peptides comprising SEQ ID NO:12 and other tumor associated rejection antigen peptides for testing in vitro or administration in vivo for any patient or tumor type.

The specification discloses that the alt.M-CSF polypeptides may be used for treating a disorder characterized by expression of an alt.M-CSF immunogenic polypeptide, for adoptive transfer, for diagnosis, for production of anti-peptide/HLA mAbs for use in imaging or purification.

Regarding design of peptides, i.e., functional variants, from motif analysis and experimental ranking algorithms the following applies. The claimed invention encompasses fragments of SEQ ID NO: 5 SEQ ID NO: 12, wherein the HLA molecule and peptide binding motif are not specified residues nor are amino acid residues not involved in MHC, i.e., the TCR contact residues. Evidentiary reference Celis et al (Molecular Immunol. 3: 1423-1430, 1994, previously provided) teach that in order to establish whether a peptide is immunogenic said peptide needs to be tested in assays that actually establish that a peptide is immunogenic. Further, although *experimental* ranking schemes are available for predicting relative binding strengths of some HLA binding peptides, and assays are available to test the binding of peptides to HLA, an undue amount of experimentation would be involved in determining peptides from the many possibilities that would be capable of binding to HLA and inducing a CTL response. Celis et al teach that "In addition to MHC binding, other factors such as antigen processing, peptide transport and the composition of the T-cell receptor repertoire could determine whether any of these peptides can function as effective CTL antigens." Evidentiary reference Ochoa-Garay et al (Molecular Immunol. 34(3): 273-281, 1997, previously provided) teach that "In summary, the results in this report indicate that the immunogenicity of a peptide cannot always be predicted from its affinity for class I or the presence of class I binding motifs. In addition, our data show that variables such as CTL

precursor frequency, peptide hydrophobicity and stability can influence the in vitro induction of CTL responses (especially page 279, last sentence and continuing onto page 280). Evidentiary reference Karin et al (J. Exp. Med. 180: 2227-2237, 1994, previously provided) teach that amino acids in an MHC binding peptide that are not the amino acids which participate in MHC binding can have a profound effect on whether or not a peptide is immunogenic. Karin et al teach that a single substitution in an amino acid, wherein said amino acid plays no role in MHC binding can completely abrogate the immunogenicity of an otherwise immunogenic peptide (especially Summary and Table 1). Thus Karin et al establish that amino acid residues not recited in the claimed peptide (e.e., amino acid residues not involved in MHC binding of a peptide) will play a pivotal role in determining whether the peptides recited in the claims are immunogenic.

Evidentiary reference Kast et al (Eur. J. Immunology 1993 23 1189-1192, previously provided) teach that the amino acid residues can exert important effects upon the binding capacity of a peptide, and hence by extension, to potential immunogenicity. Evidentiary reference DiBrino et al (J. Immunology 151(11) 5930-5935, 1993, previously provided) teach that the presence of anchor residues is not sufficient for binding to HLA because peptides with optimal amino acid residues at anchor positions failed to bind. Evidentiary reference Van der Most et al (J. Immunol. 1996, 157: 5543-5554 and Virology 1998, 240: 158-167, previously provided) teach that although an antigenic protein may contain multiple motif-fitting peptides, CTL responses are usually directed against a very limited number of immunodominant epitopes and that immunodominance appears to be determined by a variety of factors including binding affinity to HLA (and motif binding peptides bind with a wide range of affinities due to secondary anchor residues and secondary effects), intracellular processing of peptides determines whether at which level a particular peptide will be presented at the cell surface, and holes in the T cell repertoire restrict CTL responses. Van der Most et al also teach that a peptide from NP with the second highest binding affinity (IC50= 4.8nM) after the immunodominant peptide for L^d, is not recognized by LCMV-restricted CTLs. Evidentiary reference Chang et al (J. Immunol. 1999, 162: 1156-1164, previously provided) teach a peptide that was immunogenic in only a single patient despite similar HLA-binding affinity. Evidentiary reference Vitiello et al (J. Immunol. 1996, 157: 5555-5562, previously provided) teach the importance of not only binding affinity, but also of availability of specific TCRs and antigen processing in the shaping of the final repertoire of CTL specificities. Evidentiary reference Bergman et al (J Virol. 1994, 68(8): 5306-5310, previously provided) teach a discrepancy between antigenicity and immunogenicity, i.e., failure to induce CTL despite highly efficient recognition in vitro.

In addition, evidentiary reference Chaux et al (Int. J. Cancer 77 538-542, 1998, previously provided) teach that it is unclear if peptides from tumor specific proteins possessing anchor residues for binding to class I MHC produce CTL responses in patients vaccinated with the said peptides. Chaux et al further teach that detection of such CTL may require very sensitive detection assays, rather than the conventional assays disclosed in the instant specification. In

addition, Chaux et al teach that it is unclear whether the results seen in vitro are predictive of what occurs in vivo in humans.

Evidentiary reference Shastri et al (J. Immunology 1995, 155: 4339-4346, previously provided) teach that presentation of endogenous peptide/MHC class I complexes is profoundly influenced by specific C-terminal flanking residues in the peptide. The art recognizes that flanking sequences influence the processing and presentation of CTL epitopes (Eisenlohr et al, Shastri et al, Bergmann et al, Wang et al, Perkins et al, Theoboald et al and Gileadi et al) and that immunodominance can be affected by the context of the epitope within the protein molecule and that junctional neoepitopes can be created (Perkins et al), or that immunodominant epitopes can be completely silenced by contiguous sequences (Wang et al). An undue amount of experimentation would be involved in determining longer peptides from the many possibilities that would be capable of binding to HLA and being recognized by CTL. Evidentiary reference Anderton (Immunology 2001 104 367-376, previously provided) teaches that in vivo use of altered peptide ligands is unpredictable and dangerous in outbred human populations (especially paragraph spanning columns 1 and 2 on page 370). Anderton et al further teaches that to identify TCR antagonists, the need exists to generate T cell clones for in vitro analysis, with the result that often T cells are produced and are dominant in vitro that are robust enough to withstand the selective pressures of cloning, but are not representative of the entire in vivo repertoire.

It would require undue experimentation to determine which of the trillions of peptides encompassed by the claimed invention of fragments/polypeptides comprising/compositions thereof are capable of binding to an undisclosed number of HLA molecules and which immunogenic and which are not in the context of HLA/CTL combinations. Further, synthetic peptides that are chosen on the basis of scanning the protein of interest for potential peptide sequences that have a motif for binding to an HLA molecule or molecules may not induce a CTL response due to lack of Th support for CTLp to CTL.

Accordingly, there is a high level of unpredictability in designing/selecting sequences that would still maintain function, and applicant does not provide direction or guidance to do so. Because of this lack of guidance, extended experimentation that would be required to determine which substitutions/deletions/additions or permutations of amino acids would be necessary to retain activity, and it would require undue experimentation for one of skill in the art to arrive at other amino acid sequences that would have activity. In other words, since it would require undue experimentation to identify amino acid sequences that have functional activity, it would require undue experimentation to make and use the corresponding peptides. Therefore, undue experimentation would be required to determine what peptides could or could not be used in the claimed invention.

The specification does not adequately teach how to effectively use the claimed fragments/polypeptides comprising/compositions thereof capable of binding to an undisclosed number of HLA molecules and which are immunogenic in the context of HLA/CTL combinations in vivo. The specification does not teach how to extrapolate data obtained from in vitro binding and T cell stimulation assays to the development of effective in vivo human therapeutic compositions, commensurate in scope with the claimed invention. Therefore, it is not clear that the skilled artisan could predict the efficacy of the peptides exemplified in the specification and encompassed by the claims.

In view of the lack of predictability of the art to which the invention pertains, undue experimentation would be required to make and/or use the claimed invention with a reasonable expectation of success

There is insufficient guidance in the specification as to how to make and/or use the instant invention. Undue experimentation would be required of one skilled in the art to practice the instant invention. The enablement provided by the specification is not commensurate with the scope of the claims. See In re Wands 8 USPQ2d 1400 (CAFC 1988).
Applicant's arguments in the amendment filed 12/1/04 have been fully considered but are not persuasive.

Applicant's arguments are of record in the said amendment on pages 7-9.

It is the Examiner's position with regard to Applicant's arguments about a peptide comprising at least 14 consecutive amino acid residues of SEQ ID NO: 5 that does not consist of SEQ ID NO: 12, that the relevant issue is that the claims encompass a peptide comprising at least 14 consecutive amino acid residues of SEQ ID NO: 5 that does not *contain* SEQ ID NO: 12, and although the skilled artisan could make a fragment of SEQ ID NO: 5 that is at least 14 amino acid residues in length, those that do not contain the minimal epitope SEQ ID NO: 12 (a 14-mer), for those fragments the specification does not teach the use wherein the fragments do not bind to HLA-B*3501 and stimulate a CTL response. It is the Examiner's further position with regard to Applicant's arguments about an isolated polypeptide comprising the amino acid sequence of SEQ ID NO: 5 or SEQ ID NO: 12 wherein the polypeptide is not a subsequence of the alt.M-CSF protein, the claims are not limited to polypeptides comprising one of SEQ ID NO: 5 or SEQ ID NO: 12 and further comprising sequences that Applicant has provided in the specification for use in polytopes, i.e., other immunogenic peptides from other tumor rejection antigens. Although the hybrid or fusion polypeptides or polytopes can be made, the specification does not disclose use of compositions comprising polypeptides comprising SEQ ID NO: 12 and other tumor associated rejection antigen peptides for testing in vitro or administration in vivo for any patient or tumor type, and hence the skilled artisan would not know how to use the said polytopes to induce an immune response against renal carcinoma cells that present SEQ ID NO: 12, i.e., may not be relevant for use with SEQ ID NO: 12.

7. The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless -
> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

8. Claims 3, 65, and 71 are objected to as being dependent upon a rejected base claim, but would be allowable if rewritten in independent form including all of the limitations of the base claim and any intervening claims.

9. No claim is allowed.

10. Applicant's amendment necessitated the new ground(s) of rejection presented in this Office action. Accordingly, **THIS ACTION IS MADE FINAL.** See MPEP § 706.07(a). Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a). A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the date of this final action.

11. Any inquiry concerning this communication or earlier communications from the Examiner should be directed to Marianne DiBrino whose telephone number is 571-272-0842. The Examiner can normally be reached on Monday, Tuesday, Thursday and Friday.

If attempts to reach the examiner by telephone are unsuccessful, the Examiner's supervisor, Christina Y. Chan, can be reached on 571-272-0841. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

Marianne DiBrino, Ph.D.
Patent Examiner, Group 1640
Technology Center 1600
February 11, 2005

CHRISTINA CHAN
SUPERVISORY PATENT EXAMINER
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Notice of References Cited	Application/Control No. 09/786,214	Applicant(s)/Patent Under Reexamination PROBST-KEPPER ET AL.	
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	V	Wang et al. Cell. Immunol. 192 143; 284-297.
	W	Perkins et al. J. Immunol. 1991 146(7): 2137-2144.
	X	Theobald et al. J. Exp. Med. 1998 18896): 1017-1028.

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
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M. DiBrino 2/11/05

Notice of References Cited	Application/Control No. 09/786,214	Applicant(s)/Patent Under Reexamination PROBST-KEPPER ET AL.	
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	S					
	T					

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	V	
	W	
	X	

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AM. DiB 2/11/05

The Sequence Alteration Associated with a Mutational Hotspot in p53 Protects Cells From Lysis by Cytotoxic T Lymphocytes Specific for a Flanking Peptide Epitope

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Summary

A high proportion of tumors arise due to mutation of the p53 tumor suppressor protein. A p53 hotspot mutation at amino acid position 273 from R to H, flanking a peptide epitope that spans residues 264–272, renders cells resistant to killing by human histocompatibility leukocyte antigen (HLA)-A*0201–restricted cytotoxic T lymphocytes (CTLs) specific for this epitope. Acquisition of the R to H mutation at residue 273 of the human p53 protein promotes tumor growth in vivo by selective escape from recognition by p53.264–272 peptide-specific CTLs. Synthetic 27-mer p53 polypeptides covering the antigenic nonamer region 264–272 of p53 were used as proteasome substrates to investigate whether the R to H mutation at the P1′ position of the COOH terminus of the epitope affects proteasome-mediated processing of the protein. Analysis of the generated products by tandem mass spectrometry and the kinetics of polypeptide processing in conjunction with CTL assays demonstrate that the R to H mutation alters proteasomal processing of the p53 protein by inhibiting proteolytic cleavage between residues 272 and 273. This prevents the release of the natural CTL epitope that spans flanking residues 264–272 as well as a putative precursor peptide. These results demonstrate that mutation of p53 not only leads to malignant transformation but may also, in some instances, affect immune surveillance and should be considered in the design of cancer vaccines.

Key words: p53 • tumor antigens • cytotoxic T lymphocytes • antigen processing • proteasomes

Inactivation of the p53 tumor suppressor protein, through mutation or deletion, occurs in the majority of human cancers (1, 2). Often this involves a missense mutation at one of several defined mutational hotspots in the molecule (1–3). The altered p53 protein accumulates to high levels within the cell and has been used as a marker for malignant transformation (1, 2). Previous studies in both murine and human (Hu)[1] models have demonstrated the preferential susceptibility of transformed cells to lysis by CTLs that are specific for peptides representative of processed wild-type (WT) p53 protein sequences and are presented on the cell surface by class I MHC molecules (4–9). Using transgenic (Tg) mice that express the HLA-A*0201 (A*0201) molecule, we previously identified two peptide sequences from the Hu WT p53 molecule, p53.149–157 and 264–272, that bind A*0201 and serve as endogenously processed target epitopes for CTL recognition and lysis (4). Importantly, CTLs specific for these p53-derived peptide epitopes are able to recognize and lyse a broad range of p53-overexpressing and A*0201+ Hu tumor cells (4). In contrast, nontransformed cells expressing A*0201 are not killed by the same CTLs, presumably due to insufficient levels of expression of p53 and p53-derived CTL epitopes (4, 8). These peptide epitopes are also able to stimulate A*0201-restricted

[1] *Abbreviations used in this paper:* A*0201, HLA-A*0201; β_2m, β_2 microglobulin; ER, endoplasmic reticulum; Hu, human; MS, mass spectrometry; MS/MS, tandem mass spectrometry; *m/z*, mass/charge; RP, reversed phase; rVV, recombinant vaccinia virus; Tg, transgenic; WT, wild type.

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CTLs from Hu peripheral blood, suggesting the presence of CTL precursors with specificity for p53 that may be potentially mobilized to destroy tumor cells expressing high levels of p53 peptides (10–13).

Peptides that are presented on the cell surface by class I MHC molecules for recognition by CTLs most often are derived from proteolytic processing of cellular proteins by the multicatalytic proteasome complex (14–16). Proteolytic degradation of p53 has been found to be dependent on proteasomal processing (17–20). Proteasome-generated peptide products are subsequently translocated into the endoplasmic reticulum (ER) by the transporters associated with antigen processing, where they are loaded into the peptide-binding groove of nascent class I molecules (21–23). In this report, we demonstrate that proteasomal processing of the natural A*0201-restricted CTL epitope p53.264–272 and of a putative precursor peptide is profoundly affected by a mutational p53 hotspot (R to H) at the COOH-terminal flanking residue 273. As a consequence, target cells that overexpress p53 harboring the 273 R to H mutation are not susceptible to in vitro and in vivo lysis by A*0201-restricted CTLs specific for the flanking p53 epitope, 264–272. To our knowledge, these experiments demonstrate the first example of a naturally occurring mutation flanking a CTL epitope and affecting the ability of the proteasome to generate a defined MHC class I ligand.

Materials and Methods

Mice. The derivation of homozygous A2/K^b-Tg mice expressing a chimeric transgene that consists of the α1 and α2 domains of A*0201 and the α3 domain of H-2K^b has been described previously (4, 8). Mice were propagated and maintained under specific pathogen-free conditions. All experimental procedures were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Peptides. 27-mer polypeptides spanning residues 256–282 of Hu WT (TLEDSSGNLLGRNSFEVRVCACPGRDR) and mutant (273 R to H) p53 were synthesized on an automated peptide synthesizer (432A; PE Applied Biosystems, Foster City, CA). Peptides p53.264–272 (LLGRNSFEV) and 260–272 (SSGNLLGRNSFEV) were synthesized by the core facility of TSRI (430A synthesizer; PE Applied Biosystems). Purity of synthetic peptides was ascertained by reverse phase (RP)-HPLC and mass spectrometry (MS). Amino acid residues are given in single letter code.

Cell Lines. Previously described cell lines and transfectants used in these studies included T1 and T2 cells (24), the naturally A*0201-expressing, p53-deficient osteosarcoma line Saos-2, and the same cells transfected with Hu p53 genes harboring mutations at residue 143 (V to A), 175 (R to H), and 273 (R to H) (4, 25). Murine thymoma lines (EL4) transfected with A*0201/K^b (EA2K^b) or both A*0201/K^b and a Hu p53 gene harboring an R to H mutation at residue 273 (EA2K^b.1p53) have been generated as previously reported (8).

CTL Lines. The derivation and maintenance of A*0201-restricted CTL lines specific for Hu WT p53.264–272 (CTL A2 264) and p53.149–157 (CTL A2 149) have previously been described (4). CTL lines were used as effector cells at the indicated E/T ratios in ^{51}Cr-release assays (4).

Viral p53 Recombinants. The derivation of parental canarypox virus and canarypox virus p53 recombinants expressing Hu p53 without (WT) or with mutations at residue 175 (R to H) or 273 (R to H) has been previously described (5). Saos-2 targets were labeled with ^{51}Cr and infected with parental and recombinant canarypox viruses at an infectious dose of 20 PFU/cell. Cells were washed after 1 h and incubated for another 3-h period before responder CTLs were added for a 3-h ^{51}Cr-release assay (4). Recombinant vaccinia viruses (rVV) which contain minigenes encoding either p53.149–157 (rVV-ES149) or p53.264–272 (rVV-ES264) downstream of the ER insertion sequence of E19 (26), and rVV-VPE16 that expresses the gp160 gene of HIV type 1 (27), were provided by Drs. Jack Bennink and Jonathan Yewdell (National Institute of Allergy and Infectious Diseases, Bethesda, MD).

Protection of Tumor Growth in A2/K^b-Tg Mice. A2/K^b-Tg mice were either nonimmunized or immunized intravenously with 5×10^6 PFU of rVV-ES149, rVV-ES264, or rVV-VPE16. 2 wk later, mice were challenged subcutaneously in the right flank with 5×10^5 EA2K^b or EA2K^b.1p53 tumor cells. The size of tumors was determined by the formula ($a^2 \times b/2$), in which a defines the horizontal and b the vertical diameter of the tumor mass as determined by calipers. Three mice were used for each experimental and control group.

Purification of 20S Proteasomes. 20S proteasomes were purified from Hu T1 cells by standard procedures (28–31). Cell pellets were lysed in lysis buffer (80 mM KAc, 5 mM $MgAc_2$, 10 mM Hepes, pH 7.2, and 0.1% Triton X-100), dounce homogenized, and spun at 40,000 g for 20 min. Supernatant was adsorbed to equilibrated DEAE-Sephacel (Amersham Pharmacia Biotech, Piscataway, NJ) for 45 min, unbound protein was removed with wash buffer (80 mM KAc, 5 mM $MgAc_2$, and 10 mM Hepes, pH 7.2), and bound protein was eluted (500 mM KAc, 5 mM $MgAc_2$, and 10 mM Hepes, pH 7.2). The protein containing eluate was concentrated by ultrafiltration, loaded on a 10–40% sucrose gradient in wash buffer, and ultracentrifuged (40,000 rounds/min in an SW40 Ti rotor for 15.5 h; Beckman, Fullerton, CA). Gradient fractions containing 20S proteasome complexes were identified by enzyme assays using fluorogenic substrate peptides, pooled, and concentrated for chromatography on a MonoQ HR 5/5 anion exchange column (Amersham Pharmacia Biotech; eluent A: 100 mM KCl, 5 mM $MgCl_2$, and 10 mM Hepes, pH 7.2; eluent B: 1 M KCl, 5 mM $MgCl_2$, and 10 mM Hepes, pH 7.2; linear gradient). The 20S proteasomes obtained were again identified by enzymatic activity and eluted as single peak upon rechromatography. Purity of 20S proteasome preparations was >90% as assessed by Coomassie-stained PAGE gels. Two-dimensional gel electrophoresis revealed that all detectable proteasomal subunits were present in 20S proteasomes purified from the Hu T1 cell line.

In Vitro Digestion of Polypeptide Substrates by Purified 20S Proteasomes and Recognition by CTLs. 20 μg of synthetic 27-mer p53 polypeptide substrates were incubated for the indicated periods of time at 37°C with and without 1 μg of purified 20S proteasome in a total volume of 300 μl of assay buffer consisting of 20 mM Hepes-KOH, pH 7.8, 2 mM $MgAc_2$, and 1 mM dithiothreitol (28–31). ^{51}Cr-labeled T2 cells were pulsed for 30 min with degraded 27-mer peptide products at the indicated concentrations in serum-free RPMI 1640 medium supplemented with 5% vol/vol DSA and Hu β_2 microglobulin (β_2m) at 10 μg/ml. CTL A2 264 and CTL A2 149 were used as effector cells at an E/T ratio of 20:1 in a 6-h ^{51}Cr-release assay.

Peptide Analysis by MS. 10 μl of 20S proteasome degraded peptide digests were separated by a RP-HPLC SMART-System equipped with a μRPC C2/C18 SC 2.1/10 column (Amersham

Pharmacia Biotech) and eluted with a gradient of 15–65% of eluent B (70% acetonitrile in 0.05% TFA) in eluent A (0.05% TFA) in 33 min at a flow rate of 50 μl/min (29–31). Mass analysis of peptides was performed online by a tandem quadrupole mass spectrometer (TSQ 7000; Finnigan MAT, San Jose, CA) equipped with an electrospray ion source. Each scan was acquired over the range mass/charge (m/z) 300–1150 every 2 s (29–31). Peptides were identified by their molecular mass calculated according to the m/z peaks of single or multiple charged ions. Abundant peptides with signal intensities of at least threefold above background were sequenced by tandem MS (MS/MS) after fragmentation of the relevant peptides with argon atoms (30). Peptide sequence was determined from the masses of the fragmented peptide ions.

HPLC Separation of Processed Peptides, Identification by CTLs, and Sequence Analysis of Antigenic Peptides by MS/MS. 50 μl of the bulk 20S proteasome degraded peptide products were separated by RP-HPLC into 1- (50 μl) and 0.5- (25 μl) min fractions (30). ^{51}Cr-labeled T2 target cells were pulsed for 40 min with half of each of the HPLC fractions in serum-free RPMI 1640 medium supplemented with 5% vol/vol BSA and Hu β_2m at 10 μg/ml. CTL A2.264 were used as effector cells at an E/T ratio of 20:1 in a 6-h ^{51}Cr-release assay. The antigenic HPLC fractions were dried and resuspended in 50% methanol/1% acetic acid. Antigenic peptides present in the pooled HPLC fractions were identified by coelution of synthetic peptides and sequenced by MS/MS (30). Ions of m/z corresponding to the relevant double protonated peptides were fragmented by argon atoms. Collision-activated dissociation fragments of relevant m/z and derived from the pooled antigenic HPLC fractions were compared with those obtained after argon atom–mediated fragmentation of the corresponding synthetic peptides.

Extraction of Natural Peptides from Class I MHC Molecules. Adherent Saos-2/143 p53 transfectants were grown at ~10^7 cells/flask. Cells were washed twice with HBSS, and class I MHC–bound peptides were extracted by exposing cells for 1 min in 5 ml of buffer consisting of 0.13 M citric acid and 0.061 M Na_2HPO_4 at pH 3.0 (32–34). Cells were washed twice in RPMI 1640 and recultured in complete medium. Extracts were spun and the peptide containing supernatant was frozen. This procedure was repeated every other day for 3 wk to collect peptide extracts from the equivalent of 1.2×10^9 Saos-2/143 cells. Extracts were thawed, pooled, and loaded on C-18 spice cartridges (Analtech, Inc., Newark, DE) that had been washed with 4 ml each of methanol and H_2O. Cartridges were again washed with H_2O (10 ml) and peptides were eluted with 4 ml of 0.1% vol/vol TFA in acetonitrile. The peptide containing eluate was vacuum dried, resuspended in H_2O, and cleared of debris by centrifugation. The supernatant was filtered through Centricon-10 (Amicon, Beverly, MA), and the resultant peptide extract was again vacuum dried.

HPLC Separation of Natural Peptide Extracts and Reconstitution of CTL Lysis. 1 ml of resuspended natural peptide extract or 100 pmol of either the p53.264–272 or the p53.260–272 synthetic peptides were separated by RP-HPLC at a flow rate of 50 μl/min and eluted with a gradient of 20% of eluent B (70% acetonitrile in 0.05% TFA) in eluent A (0.05% TFA) in 32 min, a gradient of 20–48% of eluent B in eluent A in 74 min, and a gradient of 48–95% of eluent B in eluent A in 4 min. HPLC fractions were collected from 32 to 76 min (fractions 1–11: 4 min fractions at 200 μl/fraction) and from 76 min onwards (fractions 12–48: 1 min fractions at 50 μl/fraction). ^{51}Cr-labeled T2 target cells were pulsed for 40 min in serum-free RPMI 1640 medium supplemented with 5% vol/vol BSA and Hu β_2m at 10 μg/ml with 100 μl (fractions 4–11) or 25 μl (fractions 12–44) of fractions derived from the HPLC separation of the natural peptide extract. The corresponding volumes of fractions derived from the HPLC separations of synthetic p53.264–272 and p53.260–272 peptides and used to pulse T2 targets were 8 (fractions 4–11) and 2 μl (fractions 12–44). CTL A2 264 were used as effector cells at an E/T of 20:1 in a 5.5-h ^{51}Cr-release assay. Antigenic fractions obtained from the HPLC-separated natural peptide extract were pooled and half of the pooled natural peptides was used for rechromatography by RP-HPLC. HPLC conditions remained almost the same, yet 0.5-min fractions were collected from 56 min onwards (fractions 10–44 at 25 μl/fraction). The amount of synthetic p53.264–272 and p53.260–272 peptides used for the second HPLC separations was 60 pmol. ^{51}Cr-labeled T2 target cells were again pulsed for 40 min under serum-free conditions with 18 μl of fractions 10–32 derived from the rechromatography of the pooled antigenic natural HPLC fractions. The corresponding volumes of fractions derived from the HPLC separations of the synthetic p53.264–272 and p53.260–272 peptides and used to pulse T2 targets were 2 μl (fractions 8, 19, 20, 29) and 0.02 μl, 0.2 μl, and 2 μl (fractions 9–18 and 21–28), respectively. CTL A2 264 were used as effector cells at an E/T of 13:1 in a 6-h ^{51}Cr-release assay.

Results and Discussion

Target Cells Expressing a p53 Mutational Hotspot at Residue 273 Are Not Recognized by CTLs Specific for the Flanking Epitope 264–272. The p53-deficient Saos-2 tumor cell line transfected with a Hu p53 gene expressing the R to H hot spot mutation at amino acid residue 273 was not susceptible to lysis by CTL A2 264 specific for the flanking peptide 264–272 (Fig. 1 *A*). This contrasted with the successful recognition and lysis of cells transfected with Hu p53 genes expressing mutations at amino acid positions 143 or 175 (Fig. 1 *A*). All three p53 transfectants were killed by CTLs specific for CTL A2 149, indicating that other epitopes of the protein were being processed and presented by the A*0201 molecule (Fig. 1 *B*). Furthermore, as demonstrated in Fig. 1, *C* and *D*, Saos-2 cells infected with canarypox virus recombinants expressing Hu p53 with or without mutation at residue 175 were recognized by either CTL, whereas cells infected with a canarypox virus p53 recombinant expressing the 273 R to H mutation were lysed only by CTL A2 149 and not by CTL A2 264. Taken together, these results suggested that the R to H mutation at position 273 of the p53 protein may have prevented formation of the 264–272 peptide epitope.

Acquisition of the R to H Mutation at Residue 273 Promotes Tumor Growth In Vivo by Escape from Immune Recognition. To determine if tumors that express Hu p53 harboring the R to H mutation at residue 273 would be resistant to growth inhibition by A*0201-restricted CTLs specific for p53.264–272, A2/K^b-Tg mice were immunized with rVV strains containing minigenes encoding the sequence of either the Hu p53.149–157 (rVV-ES149) or the Hu p53.264–272 peptide (rVV-ES264). Control mice either received no vaccination or were immunized with rVV encoding the HIV type 1 envelope protein gp160 (rVV-VPE16). 2 wk later, mice were challenged with syngeneic EA2K^b or EA2K^b.1p53 transfectants, the latter of which

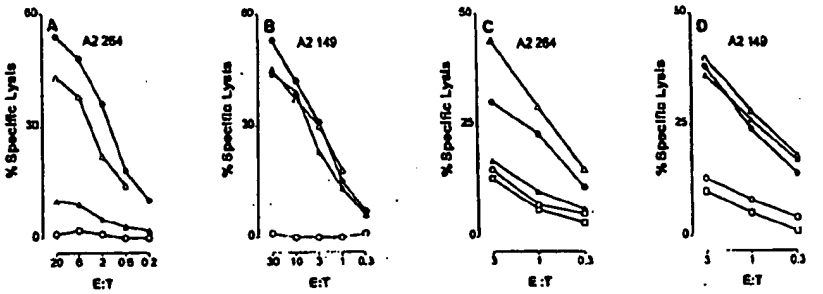

Figure 1. Cells expressing Hu p53 that contains an R to H mutation at residue 273 are resistant to lysis by A*0201-restricted CTLs specific for the flanking peptide epitope p53.264–272. A*0201-restricted CTL lines specific for peptide epitopes p63.264–272 (A2 264) (*A* and *C*) and p53.149–157 (A2 149) (*B* and *D*) were tested for cytotoxicity at the indicated E/T ratios in 5-h (*A* and *B*) and 3-h (panel *C* and *D*) ^{51}Cr-release assays. Targets in *A* and *B* were p53 deficient Saos-2 cells (○), and the same cells transfected with Hu p53 genes harboring mutations at residue 143 (V to A) (●), 175 (R to H) (Δ), and 273 (R to H) (▲). Targets in *C* and *D* were Saos-2 cells (○), the same cells infected with canarypox virus recombinants expressing Hu p53 without (●) or with mutations at residue 175 (R to H) (Δ) and 273 (R to H) (▲), and Saos-2 cells infected with the parental canarypox virus (□).

express the R to H mutation at residue 273 of Hu p53. Preimmunization with rVV-ES149 specifically prevented growth of EA2K^b.1p53 tumors, whereas vaccination with rVV-ES264 did not (Fig. 2). Vaccination with either virus led to equivalent priming of CTLs specific for the respective peptide (data not shown). Tumors that eventually grew out in rVV-ES149 preimmunized mice were found to have lost expression of Hu p53 protein (35). EA2K^b.1p53-derived tumors in untreated or rVV-VPE16–vaccinated A2/K^b-Tg mice progressed with the same growth rate as in rVV-ES264–immunized animals (35). Tumor growth of the parental EA2K^b cells, which express only low levels of murine WT p53 (8), was not prevented by either vaccination (35). These results indicate that tumors harboring the R to H mutation at residue 273 of the Hu p53 protein are able to selectively escape recognition in vivo by CTLs specific for the flanking peptide 264–272.

Peptides Derived from 20S Proteasome Degradation of the Synthetic 27-mer Polypeptide p53.256–282 Carrying an R to H Mutation at Residue 273 Are Not Recognized by CTL A2 264. In several reports, it has been demonstrated that the proteasome system represents a major source for the generation of MHC class I ligands (14–16, 28, 36). As demonstrated previously, degradation of p53 is dependent on proteasomal processing (17–20). Accordingly, one mechanism that could interfere with the availability of the 264–272 epitope would be the inability of the proteasome to produce this peptide from the 273 R to H mutant p53. This could arise if the COOH-terminal cleavage site of the 264–272 epitope was abrogated by the change from R to H at the epitope flanking residue 273. To test this hypothesis, synthetic 27-mer p53 peptides spanning residues 256–282 and containing the 264–272 epitope flanked by either the WT or mutant residue at position 273 were used as substrates for in vitro digestion by purified 20S proteasomes. T2 target cells that express predominantly empty A*0201 molecules (24) were pulsed with the peptide digests and tested for recognition by CTL A2 264. Substantial lysis of T2 targets was observed when cells were pulsed with peptides derived from the 20S proteasome–degraded WT 27-mer, but not with those derived from degraded mutant 27-mer (Fig. 3). Recognition of the synthetic 9-mer p53.264–272 epitope as compared with the WT p53.256–282 peptide digest was 2.4 logs more efficient (2.3–2.7 logs as compared with three independent WT 27-mer peptide digests), sug-

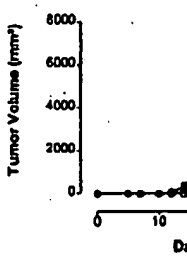

Figure 2. Immunization of A2/K^b-Tg mice with the p53.264–272 epitope does not prevent growth of tumor cells expressing both A*0201/K^b and Hu p53 containing an R to H mutation at residue 273. A2/K^b-Tg mice were immunized intravenously with 5 × 10^6 PFU of rVV-ES149 (○) or rVV-ES264 (●). 2 wk later (day 0), mice were challenged subcutaneously in the right flank with 5 × 10^5 EA2K^b.1p53 (273 R to H) tumor cells. Nontreated or rVV-VPE16–vaccinated (encoding the HIV type 1 envelope protein gp160) A2/K^b-Tg mice behaved the same as rVV-ES264–immunized animals; tumors appeared on day 10 after challenge and progressed with the same growth rate. These negative controls are not shown for clarity.

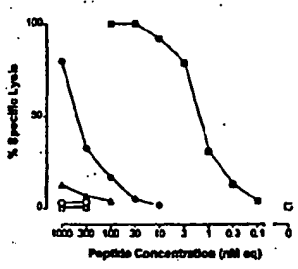

Figure 3. Peptide products derived from in vitro 20S proteasome degradation of the synthetic 27-mer polypeptide p53.256–282 carrying an R to H mutation at residue 273 are not recognized by CTLs specific for the nonameric p53.264–272 peptide epitope. Synthetic 27-mer polypeptide substrates spanning residues 256–282 of WT (TLEDSSGNLLGRNSFEVRV-CACPGRDR) or mutant (273 R to H) p53 were incubated for 24 h with and without purified 20S proteasome. ^{51}Cr-labeled T2 cells were pulsed under serum-free conditions with the indicated concentrations of WT 27-mer products that had been incubated with (●) and without (○) 20S proteasome, mutant 27-mer products that had been incubated with (▲) and without (Δ) 20S proteasome, the synthetic p53.264–272 peptide epitope (■), or no peptide (□). A*0201-restricted CTLs specific for the p53.264–272 peptide epitope were used as effector cells at an E/T ratio of 20:1 in a 6-h ^{51}Cr-release assay. Peptide concentration is given as the equivalent of the input concentration of 27-mer polypeptide substrates before proteasomal degradation (*nM eq*). Peptide-pulsed T2 targets were not lysed by CTLs specific for the unrelated p53.149–157 peptide (data not shown).

gesting that the 264–272 epitope did not represent an abundant product generated from the WT 27-mer by the 20S proteasome. The selective recognition of WT as opposed to mutant 27-mer peptide digests by CTL A2 264 was dependent on degradation by the 20S proteasome as no lysis was detectable when WT and mutant 27-mer peptides were incubated in the absence of the 20S proteasome. Kinetic studies revealed that WT degradation products recognized by T cells became detectable after 1 and 2 h of incubation of the polypeptide with purified 20S proteasome (Fig. 4, *A* and *B*), reached a peak of activity after a period of between 5 and 8 h of proteasomal cleavage (Fig. 4, *C* and *D*), and could still be observed after 27 h of proteasome incubation (data not shown). These results indicate that the R to H mutational change at residue 273 affected the ability of the proteasome to release an antigenic peptide from the 27-mer that could be recognized by CTL A2 264.

The p53 Mutation at Residue 273 Abrogates the Proteasomal Cleavage Site between p53 Residues 272 and 273. To prove that the COOH-terminal cleavage site of the 264–272 epitope has indeed been abrogated by the change from R to H at flanking residue 273, the 20S proteasome–degraded (24-h) WT and mutant p53.256–282 peptide products were separated by RP-HPLC, abundant masses were identified online by MS, and the sequence of abundant peptides was confirmed by MS/MS. The majority of the dominant cleavage products generated by the 20S proteasome from the WT and mutant 27-mer polypeptides were comparable in sequence and quantity (Fig. 5, *A* and *B*). However, within the range of signal intensities of at least threefold above background, only two peptides, 260–272 and 256–272, both of which contained the COOH-terminal residue 272 of the CTL epitope 264–272, were observed (Fig. 5 *A*). Notably, the relative signal intensities of peptides 260–272 and 256–272 were found to be 14- and 20-fold higher, respectively, within the WT as opposed to the mutant peptide digests (Fig. 5, *C* and *D*, panels *2* and *3*). The differential detectability of these peptides within the WT as opposed to the mutant 27-mer peptide digests was not due to differences in the efficiency of proteasomal degradation of either polypeptide. In general, the efficiency of 20S proteasome–induced degradation of either 27-mer peptide was >95% (Fig. 5, *C* and *D*, panel *4*). Also, comparable amounts (relative signal intensity: twofold more in mutant versus WT) of the peptide 266–275, which did not use the relevant cleavage site between residues 272 and 273, were generated from the WT and mutant 27-mer (Fig. 5, *C* and *D*, panel *1*).

These results indicate that the p53 R to H mutation at residue 273 retards proteolytic cleavage by the 20S proteasome between residues 272 and 273. However, we could not detect the peptide representing the CTL epitope 264–272. The proteolytic generation of the 264–272 epitope from the WT 27-mer at a quantity below the threshold of detectability by MS could have been responsible for such lack of detection, even though the bulk WT 27-mer degradation products had been recognized by CTL A2 264. The observation that the 266–275 peptide was an abundant proteasomal cleavage product of both the WT and mutant 27-mer substrate would support this interpretation, as the release of this peptide would interfere with the generation of the 264–272 CTL epitope (Fig. 5). However, it could also be possible that the peptide 260–272, which used the COOH-terminal cleavage site between residues 272 and 273, represents a precursor peptide that is subsequently trimmed in the cytosol or the ER to the size of the optimal antigenic CTL epitope 264–272. Postproteasomal trimming of the NH_2 terminus of precursor peptides has been observed previously (31, 37–40).

The p53.264–272 CTL Epitope Is Generated by Proteasomal Degradation of the WT as Opposed to the Mutant 27-mer Polypeptide Substrate. To improve the sensitivity of detection of the 264–272 peptide, the proteasome degraded peptide products (24-h) were fractionated by RP-HPLC. T2 targets were sensitized with the individual HPLC fractions and tested for recognition by CTL A2 264. Only T2 cells that had been pulsed with fractions 24, 25, and 26 of the WT 27-mer peptide digests were recognized by CTL A2 264 (Fig. 6, *A* and *B*). The retention time of fraction 24 was matched by that of the synthetic 264–272 peptide (data not shown). WT fractions 24 and 25 were pooled and tested for the presence of the 264–272 peptide by MS/MS. Identical collision-activated dissociation fragments of ions corresponding to the double protonated 264–272 peptide were detected in the pooled WT fractions 24 and 25, and the synthetic 264–272 peptide (Fig. 6, *C* and *D*). The relevant collision-activated dissociation fragments were absent

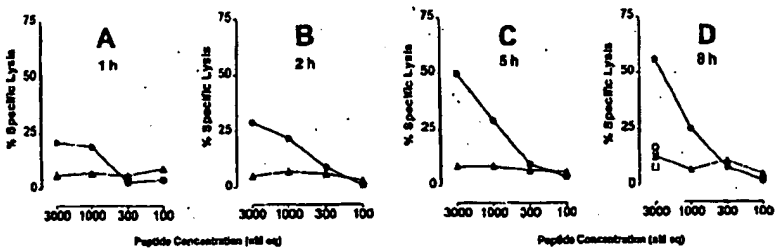

Figure 4. Kinetics of 20S proteasomal degradation of WT and mutant 27-mer p53 polypeptide substrates. WT and mutant p53.256–282 peptide substrates were incubated for 1 (*A*), 2 (*B*), 5 (*C*), and 8 (*D*) h with and without purified 20S proteasome. ^{51}Cr-labeled T2 cells were pulsed under serum-free conditions with the indicated concentrations of WT 27-mer products that had been incubated with (●) and without (○) 20S proteasome, and mutant 27-mer products that had been incubated with (▲) and without (△) 20S proteasome. Material derived from the incubation of 20S proteasome in the absence of any polypeptide substrate served as another negative control (□). CTL A2 264 were used as effector cells at an E/T ratio of 20:1 in a 6-h ^{51}Cr-release assay. Peptide concentration is given as the equivalent of the input concentration of 27-mer polypeptide substrates before proteasomal degradation (*nM eq*).

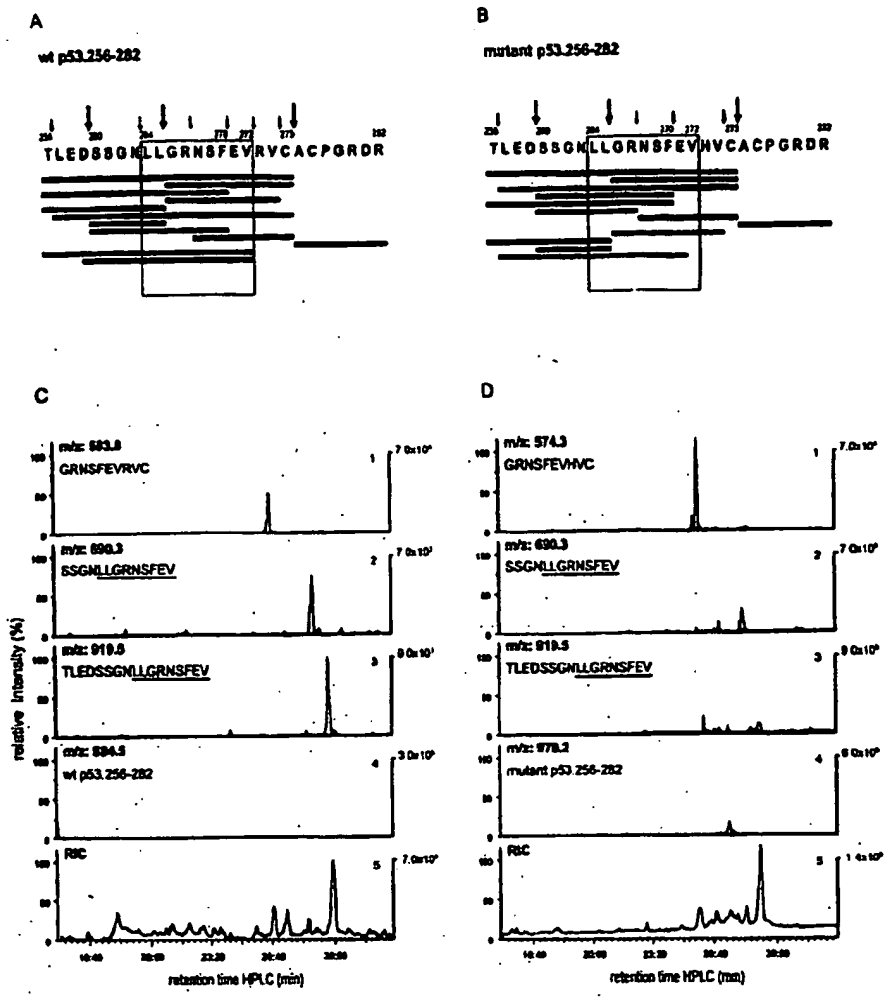

Figure 5. The p53 hotspot mutation at residue 273 (R to H) abrogates the proteasomal cleavage site between p53 residues 272 and 273. Bulk peptide products derived after 24 h from 20S proteasome–mediated degradation of the synthetic WT (*A* and *C*) and mutant (273 R to H) (*B* and *D*) 27-mer polypeptides p53.256–282 covering the A*0201-restricted CTL epitope p53.264–272 (LLGRNSFEV) were separated by RP-HPLC and analyzed online by MS. Abundant peptide products were sequenced by MS/MS. Cleavage products with signal intensities of at least threefold above background and identified by mass (MS) and sequence (MS/MS) are shown in descending order according to their signal intensities (*A* and *B*, *black bars*). The amino acid sequence of WT (*A*) and mutant (*B*) peptide substrates with abundant (*large arrows*) and nonabundant (*small arrows*) cleavage sites is also presented. The small broken arrow (*A*) represents the theoretical NH_2-terminal cleavage site of the nonameric CTL epitope 264–272. The quantitative comparison of some of the relevant WT (*C*) and mutant (*D*) cleavage products is shown by their elution profiles and relative signal intensities as measured by the ion current of double protonated peptide ions. As demonstrated in *C* and *D*, peptide 266–275 (GRNSFEV-*R*/*H*-VC) represents a dominant product that interferes with the formation of the 264–272 CTL epitope (panel *1*). The WT p53 peptides 260–272 (SSGNLLGRNSFEV) and 256–272 (TLEDSSGNLLGRNSFEV) use the COOH-terminal cleavage site of the minimal CTL epitope 264–272 between WT residues 272 and 273 (panels *2* and *3*). Panel *4* shows the uncleaved WT and mutant 27-mer substrate peptides left after proteasomal degradation and used for adjusting the scale (percentage of relative intensity). Panel *5* gives the total ion current of the bulk proteasomal degradation products.

in pooled WT fractions 22 and 23, and in pooled mutant fractions 22 and 23 and 24 and 25 (data not shown). These results indicate that the p53.264–272 CTL epitope had been generated directly from the WT 27-mer by the 20S proteasome. Its low abundance is not unique, but a property shared with immunodominant nonameric CTL epitopes detected in 20S proteasome in vitro digests (30, 41–45). CTL assays of p53-deficient Saos-2 cells infected with rVV expressing minigenes encoding either the 149–157 or the 264–272 peptide without an ER insertion sequence revealed that the 264–272 peptide is efficiently translocated into the ER (Theobald, M., and L.A. Sherman, unpublished observation). These findings demonstrate that the p53 hotspot mutation from R to H at residue 273 prevents the 20S proteasome–mediated COOH-terminal cleavage of the flanking peptide 264–272 and its subsequent presentation by A*0201.

The more abundant flanking peptide 256–272 eluted in WT fraction 26, which was recognized poorly by CTL A2 264 (Figs. 5 *A* and 6 *A*), making it unlikely to be an independent CTL epitope. However, it is of interest that due to the HPLC conditions used, the other abundant flanking peptide 260–272 (Fig. 5 *A*) was identified by MS/MS to be present in the pooled WT fractions 24 and 25 (data not shown). However, the A*0201-binding affinity of the longer peptide 260–272 was 2.5-fold lower than that of the 9-mer 264–272, and it was found to be recognized 30–100-fold less efficiently by CTL A2 264 (data not shown). These results favor the view that 260–272 may represent a precursor peptide that is eventually trimmed in the cytosol or the ER to the 9-mer rather than functioning as an independent CTL epitope.

*The Hu WT p53.264–272 Peptide Is the Natural Epitope Presented by A*0201 and Recognized by CTL A2 264.* To

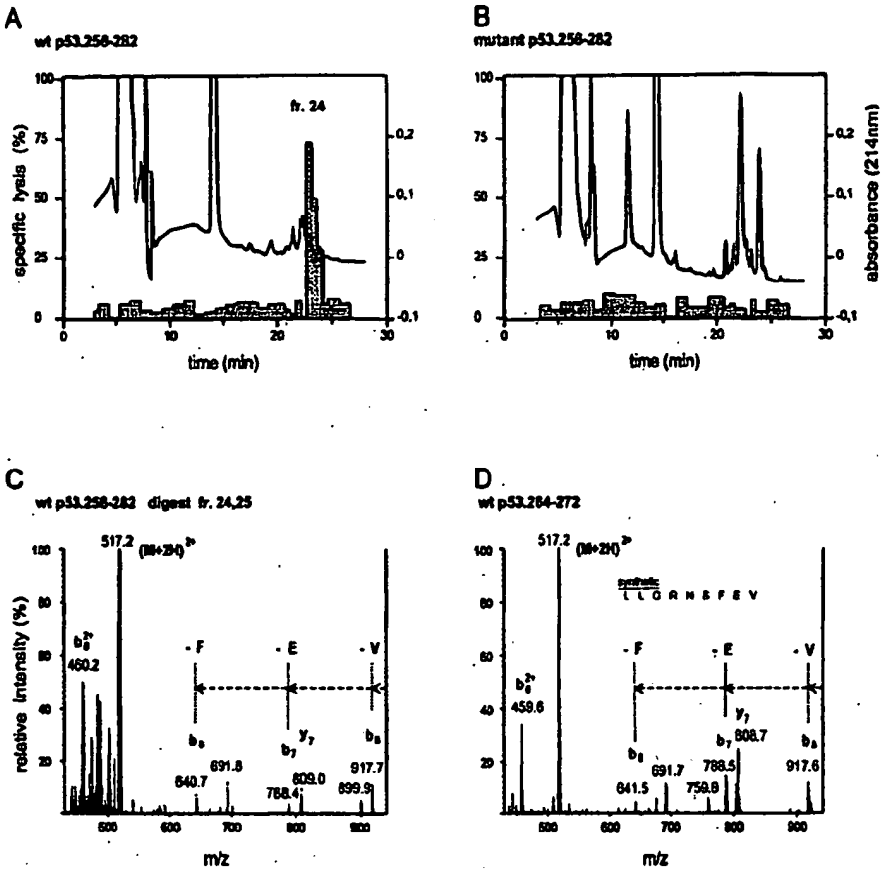

Figure 6. The p53.264–272 CTL epitope is generated by proteasomal degradation of the WT as opposed to the mutant 27-mer p53.256–282 polypeptide substrate. Bulk 20S proteasome-degraded (24-h) WT and mutant 27-mer peptide products were fractionated by RP-HPLC. ^{51}Cr-labeled T2 target cells were pulsed for 40 min under serum-free conditions with half of each of the WT and mutant HPLC fractions and tested for susceptibility to lysis by CTL A2 264 at an E/T ratio of 20:1 in a 6-h ^{51}Cr-release assay. The HPLC profile (absorbance: —) and the specific lysis (*shaded columns*) of T2 targets sensitized with individual WT (*A*) and mutant (*B*) HPLC fractions is shown. WT fractions 24 and 25 were pooled and tested by MS/MS for detection of the p53.264–272 (LLGRNSFEV) peptide epitope. Ions of m/z = 517.2 corresponding to the double protonated 264–272 9-mer peptide were fragmented by argon atoms. Collision-activated dissociation fragments of m/z 517.2 and derived from the pooled WT fractions 24 and 25 (*C*) were compared with those obtained after argon atom–mediated fragmentation of the synthetic 264–272 peptide (*D*). In particular, fragments b8, b7, and b6, lacking the COOH-terminal residues V, E, and F, respectively, were detectable in both the pooled WT fractions 24 and 25 (*C*) and the synthetic 264–272 9-mer peptide (*D*).

identify the natural peptide epitope presented by A*0201 and recognized by CTL A2 264, peptides were extracted from class I MHC molecules of Saos-2/143 p53 transfectants. As the 9-mer (264–272) and 13-mer (260–272) p53 peptides had an almost identical retention time under the HPLC conditions used for purifying the 20S proteasomal peptide digests, a shallower elution gradient was applied for the RP-HPLC fractionation of natural peptide extracts as well as synthetic 9-mer and 13-mer peptides. The pool of natural peptides extracted from Saos-2/143 cells was chromatographed by HPLC and 4-min fractions collected and assayed. To obtain final resolution, antigenic fractions 7 and 8 were pooled and half of the pool was rechromatographed. At the relevant retention time, 0.5 min fractions were collected in order to discriminate between the 264–272 and the 260–272 peptides. T2 cells sensitized with individual HPLC fractions served as targets for CTL A2 264. As shown in Fig. 7 *A*, the peak of CTL activity obtained after rechromatography of fractions 7/8 and derived from the natural peptide extract was detected in fraction 12. This HPLC fraction had the same conductivity and retention time as compared with the antigenic fractions (12 and 13) derived from the HPLC separation of the synthetic 264–272 9-mer peptide (Fig. 7 *B*). In contrast, the antigenic activity of the synthetic 260–272 13-mer peptide eluted in fraction 24 and its retention time differed from the 264–272 peptide by .6 min under the conditions used for rechromatography (Fig. 7 *C*). The lower amount of lysis obtained with fraction 24 of the synthetic 260–272 peptide again indicated less efficient crossrecognition by CTL A2 264. Pulsing T2 targets with a 10-fold higher amount (2 μl) of fraction 24 of the synthetic 260–272 peptide resulted in a substantial increase of lysis (100% specific lysis by CTL A2 26; (data not shown). However, as little as 0.02 μl of either fraction 12 or 13 of the synthetic 264–272 peptide was sufficient to detect substantial lysis (fractions 12 and 13: 50 and 61% specific lysis, respectively) of peptide-pulsed T2 cells by CTL A2 264 (data not shown). No antigenic activity could be observed with fraction 24 after rechromatography of the natural peptide extract (Fig. 7 *A*). These results indi-

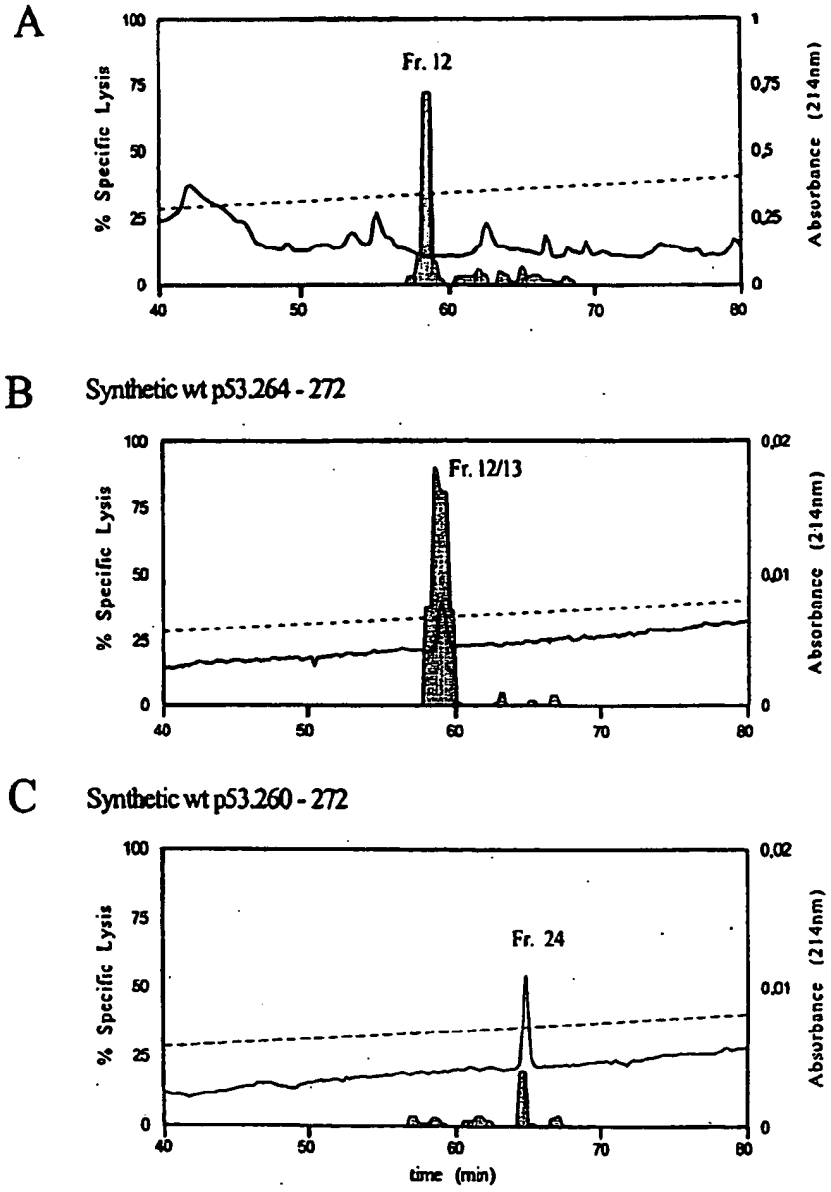

Figure 7. The Hu WT p53.264–272 peptide is the natural epitope presented by A*0201 for recognition by CTL A2 264. Natural peptides were extracted from class I molecules of Saos-2/143 p53 transfectants. Synthetic p53.264–272 and 260–272 peptides and the natural peptide extract were fractionated by RP-HPLC. Individual HPLC fractions were used to sensitize T2 targets and to reconstitute lysis by CTL A2 264. Two antigenic HPLC fractions obtained from the natural peptide extract had an almost identical retention time and conductivity as compared with those antigenic HPLC fractions derived from the synthetic p53.264–272 and p53.260–272 peptides. As 4-min HPLC fractions were collected at the relevant retention time, the peak of CTL activity occurred in almost identical HPLC fractions, although the retention time of the synthetic p53.264–272 and p53.260–272 peptides differed from each other by ~4 min. To separate either peptide from one another and identify the natural antigenic peptide, half of the pooled antigenic fractions of the HPLC-separated natural peptide extract (*A*) as well as 60 pmol of synthetic 264–272 (*B*) and 260–272 (*C*) peptides were used for rechromatography by RP-HPLC. HPLC conditions remained almost the same, yet 0.5-min fractions (25 μl/fraction) were collected at the relevant retention time in order to discriminate between the 264–272 and the 260–272 peptides. ^{51}Cr-labeled T2 target cells were pulsed for 40 min under serum-free conditions with individual HPLC fractions (fractions derived from the rechromatography of natural peptides: 18 μl; fractions 8, 19, 20, and 29 derived from synthetic p53 peptides: 2 μl; fractions 9–18 and 21–28 derived from synthetic p53 peptides: 0.02 μl, 0.2 μl, and 2 μl) and tested for susceptibility to lysis by CTL A2 264 at an E/T of 13:1 in a 6-h ^{51}Cr-release assay. The HPLC profile (absorbance: —) (*A*: first HPLC separation of natural peptide extracts) and the specific lysis (*shaded columns*) of T2 targets sensitized with individual HPLC fractions obtained from the rechromatography of natural peptides (*A*), 2 μl (fractions 8, 19, 20, and 29), and 0.2 μl (fractions 9–18 and 21–28) of HPLC fractions obtained from the synthetic p53.264–272 (*B*) and p53.260–272 (*C*) peptides is shown.

cate that the Hu WT p53.264–272 as opposed to the longer 260–272 peptide is the natural epitope presented by A*0201 and recognized by CTL A2 264. However, as the 13-mer 260–272 (and the 17-mer 256–272) peptide(s) are the more abundant products released from the WT p53 sequence by the 20S proteasome (Fig. 5, *A* and *C*), it is possible that these peptides undergo NH_2-terminal trimming in the cytosol or ER (37–40) and represent precursor peptides that may thereby contribute substantially to the availability of the low-abundant WT p53 processing product 264–272 for its assembly with nascent A*0201 molecules in the ER.

Several recent studies have focused on the mutational alteration within a peptide epitope to demonstrate a way by which viruses can escape recognition by epitope-specific CTLs (46–50). This is generally believed to involve peptide binding to the MHC class I molecule, or recognition by the TCR (46–50). However, in a recent report it was shown that a mutation within a viral peptide epitope affected the production of precursor peptides by proteasomal degradation through the introduction of a dominant new proteasomal cleavage site within the viral epitope (31). Based on studies that evaluated the effect of synthetically altering residues

flanking a peptide epitope on its processing, it would be predicted that point mutation outside of the epitope could also interfere with its presentation (30, 36, 44, 51–56). To our knowledge, our studies represent the first report of such a naturally occurring mutation outside the epitope that alters CTL recognition of a flanking peptide. In fact, the single mutation described here interferes not only with the proteasomal processing of putative precursor peptides, but also with the generation of the optimal CTL epitope itself. It is at present unclear why the R to H mutation interferes with the COOH-terminal proteasomal cleavage. It is possible that a change in charge and size of the flanking residue influences the COOH-terminal cleavage site used by the proteasome. However, as the possibility that a particular site will be preferred for proteasomal cleavage is dependent on amino acid sequences within the epitope (31, 51), as well as on flanking residues both up- and downstream (30, 36, 44, 51–56), it is likely that the rules by which ligand generation is governed are more complex and not yet understood.

Treatment of cells with IFN-γ has been reported to alter the processing kinetics, quantity, and quality of MHC class I–bound peptide ligands by affecting the expression both of the IFN-γ–inducible proteasomal subunits (LMP2, LMP7, and MECL-1) and the IFN-γ–inducible PA28-α/β activator complex (11S regulator) of the 20S proteasome (28, 29, 41, 43, 57–69). Exposing Saos-2/273 (R to H) p53 transfectants to IFN-γ (10 ng/ml for 20 h) resulted in an only partial reconstitution of lysis by CTL A2 264 (20% specific lysis of nonpretreated Saos-2/273 cells versus 31% specific lysis of IFN-γ pretreated Saos-2/273 targets at an E/T of 20:1) (Häussler, A., and M. Theobald, unpublished observation). The molecular basis of this IFN-γ–mediated partial reconstitution of Saos-2/273 killing by CTL A2 264 on the level of both the proteasomal subunit composition (LMP2, LMP7, and MECL-1) and the PA28 activator (11S regulator) expression is currently under investigation.

Based on these results, it may be concluded not only that the p53 mutation at residue 273 is associated with malignant transformation, but that it can also affect CTL recognition in vitro and in vivo of tumor cells carrying this mutation. It is tempting to speculate that cells harboring this mutation may have a competitive edge for growth in A^*0201^+ individuals by evading CTL recognition (70). Several other reports have shown that vaccination of mice with the intact p53 protein expressed in a viral vector or with p53 peptides pulsed onto dendritic cells can prevent growth in vivo of tumors expressing high levels of p53 (5, 7). Furthermore, recent studies have demonstrated the ability of p53.264–272 epitope–specific Hu CTL to lyse squamous cancer cells (6). Thus, knowledge not only of the antigenic epitopes of p53, but also of the biological role of their sequence context and its modulation by frequently arising mutations may explain disease progression, and may assist in the design of efficacious cancer vaccines.

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Differential Effects of Flanking Residues on Presentation of Epitopes from Chimeric Peptides

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Chimeric peptides in which the optimal *H-2*^{d} mouse hepatitis virus nucleocapsid (pN) and human immunodeficiency virus type 1 (p18) epitopes, separated by 38, 7, or 2 amino acids, were expressed from a single open reading frame by using recombinant vaccinia viruses to analyze antigen processing of proximal class I-restricted epitopes. Recognition of the carboxy-terminal *D*^{d}-restricted p18 epitope was independent of the amino-terminal flanking residues. By contrast, proximity of the carboxy-terminal epitope decreased recognition of the amino-terminal *L*^{d}-restricted pN epitope. Immunization resulted in the induction of both p18- and pN-specific antiviral cytotoxic T lymphocytes, irrespective of the number of amino acids separating the epitopes.

Cytotoxic T lymphocytes (CTL) recognize processed antigen in the form of 8- to 10-amino-acid (aa) peptides in association with major histocompatibility complex (MHC) class I molecules (10, 18, 21). These peptides are generally derived from antigens which are proteolytically degraded in the cytosol and transported into the endoplasmic reticulum, where they assemble with class I heavy chains and β2 microglobulin to form a stable tripartite complex which is transported to the plasma membrane (11, 16, 21). Naturally processed class I binding peptides are characterized by a sequence motif specific for the allelic variations within the cleft of individual class I heavy chains (18, 21). However, despite the presence of numerous peptides with potential binding motifs in an antigen, CTL responses to only a very limited number of determinants are induced (2, 17, 28). Although the cytosol and the endoplasmic reticulum are sources for peptides (11), it is unclear whether class I molecules associate with mature peptides that have undergone cleavage to the optimal epitope or assemble with larger precursor peptides, which are trimmed during or after assembly (21). The highly selective cell surface presentation of individual peptides is regulated primarily by peptide-class I binding affinity (10, 11, 17, 28). However, accessibility to proteolysis and specificity of peptide transporter proteins provide additional limiting factors for peptide presentation (6, 11, 19, 22).

The primary role of residues within the CTL epitope for class I presentation has clearly been established (10, 13, 17, 28) and is supported by efficient presentation of endogenous epitopes lacking flanking sequences (2, 3, 8) or placed in the context of heterologous or mutated flanking residues (5–7, 12, 13, 27). The influence of flanking sequences on class I processing, however, remains controversial. Alterations in the 5 to 10 residues localized directly adjacent to an optimal epitope may cause defective presentation, as demonstrated by the reduced or inhibited presentation of natural *L*^{d}- and *K*^{d}-restricted determinants (7, 8, 13).

Efficient processing has implications for both the outcome of a CTL response following viral infection and the design of recombinant polyvalent vaccines containing multiple T-cell epitopes to provide protective immunity (6–8, 27). Several problems concerning class I antigen presentation may arise when multiple epitopes are linked. First, residues from one epitope may form flanking sequences deleterious to the presentation of adjacent epitopes. Second, the differential trafficking rate of individual class I molecules (1) may result in preferential induction of CTL specific for the epitope that is presented by a faster-trafficking class I molecule. Third, processing of the chimeric peptides may result in the formation of novel epitopes which may induce CTL that do not recognize native antigen (20).

To establish a model for analysis of antigen presentation and CTL induction within the *H-2*^{d} haplotype, we tested responses to two immunodominant viral epitopes expressed as chimeric peptides: one epitope comprises the *D*^{d}-restricted p18 sequence from the gp160 protein of human immunodeficiency virus type 1 (HIV-1) strain IIIB (26); the second epitope, designated pN, is *L*^{d} restricted and derived from the nucleocapsid protein (JN) of the JHM strain of mouse hepatitis virus (MHV; 2). The optimal peptides are a 10-mer (aa 318 to 327, p18-10) within the p18 epitope (3) and a 9-mer (aa 318 to 326, pN318-326) within the pN epitope (2). Both epitopes are efficiently recognized when expressed endogenously in either the presence (peptides comprising 51 to 67 aa) or the absence of native flanking sequences (9 or 10 aa) by CTL specific for native antigen (2, 3).

The influence of epitope proximity on presentation was investigated by using three minigenes expressing tandem peptides in which the HIV p18 and MHV pN epitopes were separated by 38 (tan38)-, 7 (tan7)-, and 2 (tan2)-aa spacers (Fig. 1). Immediate flanking sequences of both epitopes in tan38 and tan7 are composed of native sequences (Fig. 1); however, the p18-10 epitope in tan2 is directly linked to native JN residues. Genes encoding tan38, tan7, and tan2 with an initiation start codon and stop codons were cloned into vaccinia virus (Vac) vectors pTM1 (9) and pSC11ss-derived pK (3). Homologous recombination with the wild-type Vac WR strain as previously described (25) resulted in Vac recombinants vtan38, vtan7, and vtan2. The gene encoding tan38 was

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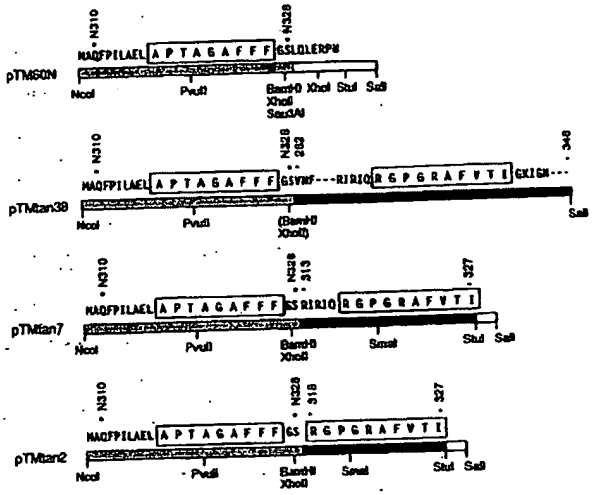

FIG. 1. Schematic of the minigenes and amino acid sequences of chimeric peptides. Minigenes are represented by bars. JN-specific coding sequences are shaded, gp160 is in black, and the vector-derived polylinker is in white. Restriction site positions are shown relative to the peptide sequence. Amino acids are in the one-letter code; the boxed amino acids represent the optimal peptide required for CTL recognition. The beginning and end of JN- and gp160-derived residues are marked by the symbols * and ^, respectively. The numbering of amino acids identifies their position in the native proteins. Designations of the chimeric constructs indicate the lengths of intervening flanking residues between the epitopes.

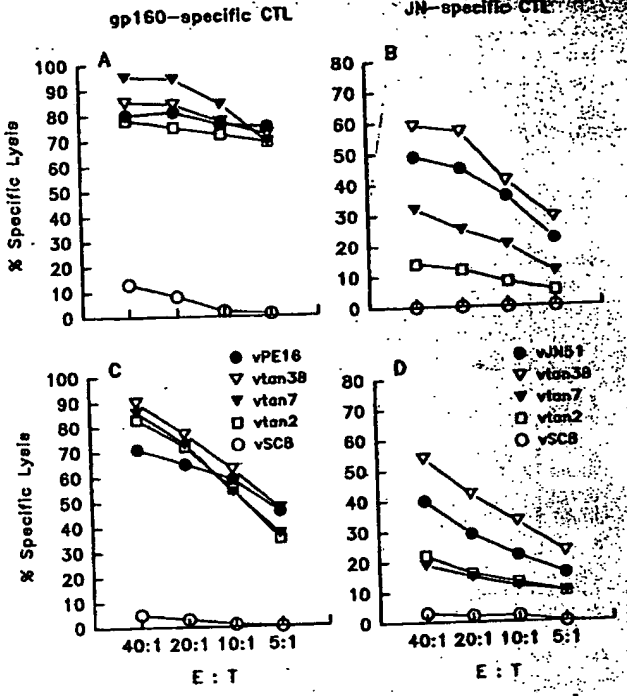

FIG. 2. Differential recognition of epitopes derived from endogenous chimeric peptides. J774.1 targets ($H\text{-}2^d$) (A and B) or L929 target cells ($H\text{-}2^k$) expressing either D^d (C) or L^d (D) molecules were infected with either vtan38, vtan7, or vtan2. Targets infected with Vac recombinants expressing either gp160 (vPE16) or aa 301 to 355 of the JN protein (vJN51) served as positive controls; vSC8 (expressing the *Escherichia coli lacZ* gene)-infected targets were negative controls. Presentation of p18 was tested with gp160-specific CTL (A and C), and that of pN was tested with JN-specific CTL (B and D). Symbol legends are identical for panels A and C and panels B and D. Cytolytic activity was measured in a 4-h ^{51}Cr release assay as previously described (2, 3). Effector cells were added at the effector/target cell ratios (E:T) indicated. The cytolytic activity shown is representative of three independent assays.

assembled by sequential cloning of a 64-bp fragment encoding JN aa 310 to 328 and a 210-bp fragment encoding gp160 aa 282 to 348 into pTM1 (9). The JN minigene was amplified by PCR as previously described (2), with primer oligonucleotides ON1011 (5′-ATA GGA TCC ATG GCA CAG TTC CCC ATT CTT GCA; nucleotides 1011 to 1026) and ON1427 (5′-TTA CCC GGG CAC ATT AGA GTC ATC TTC TAA C; nucleotides 1427 to 1447), which contained *Nco*I and *Sma*I sites to introduce an ATG start codon and allow unidirectional cloning. Following digestion with *Nco*I and *Sau*IIIA, the PCR product was cloned into the *Nco*I-*Bam*HI sites of pTM1 to yield plasmid pTM60N. Plasmid pTMtan38 was obtained by insertion of an *Xho*II-*Sal*I fragment encoding the gp160 V3 loop (3) into the *Bam*HI-*Sal*I sites of pTM60N. Minigene tan38 was excised from pTMtan38 with *Nco*I and *Hin*cII and ligated into the *Nco*I-*Stu*I sites of plasmid pK (3), resulting in plasmid pKtan38. Construct tan2 was generated by direct cloning of complementary oligonucleotides CB7 (5′-GATCCA GAG GAC CCG GGA GAG CAT TTG TTA CAA TAT AGG and CB10 (5′-CCT ATA TTG TAA CAA ATG CTC TCC CGG GTC CTC TG) into the *Bam*HI-*Stu*I sites of plasmid pTM60N downstream of the pN epitope minigene. The resulting plasmid, pTMtan2, contained a unique *Sma*I site within the coding region of the p18-10 epitope and a stop codon introduced by the oligonucleotides. Construct pTMtan7 was generated by inserting annealed oligonucleotides CB8 (5′ GAT CCA GAA TCC GTA TCC AGA GAG GAC CC) and CB9 (5′ GGG TCC CCT CTG GAT ACG GAT TCT G) encoding the 7-aa spacer region into the *Bam*HI-*Sma*I sites of pTMtan2. Plasmids pKtan2 and pKtan7 were generated by subcloning the respective *Nco*I and *Stu*I tan7 and tan2 fragments into the *Nco*I-*Sma*I sites of plasmid pK. Sequences were confirmed by using pSC11ss-specific primers as previously described (25).

The ability of the chimeric peptides to be processed for simultaneous presentation by both the D^d and L^d class I molecules was assessed by using J774.1 ($H\text{-}2^d$) cells. Targets were infected with Vac recombinants vtan38, vtan7, and vtan2 at a multiplicity of 5, incubated for 12 h at 37°C, and tested for recognition of the individual epitopes by both gp160 (Fig. 2A)- and JN (Fig. 2B)-specific CTL in a ^{51}Cr release assay as previously described (2, 3). Antigen-specific CTL populations were in vitro-restimulated cultures derived from BALB/c mice immunized with either JHM or a Vac recombinant expressing gp160 (vPE16) as previously described (2, 3). In numerous experiments, the p18 epitope was recognized equally well by gp160-specific CTL independent of the amino (N)-terminal sequences (Fig. 2A). However, parallel analysis of identical targets consistently showed a loss of pN-specific recognition with increased proximity to the carboxy (C)-terminal p18 epitope (Fig. 2B). To rule out a processing defect or preferential binding of the chimeric precursor peptide to D^d as a cause for decreased L^d-restricted presentation in $H\text{-}2^d$ cells, L929 cells ($H\text{-}2^k$) expressing either L^d (K2a7) or D^d (K8-30) class I molecules were tested. Figure 2C and D confirms the consistently higher levels of p18 recognition on D^d and the impaired presentation of the pN epitope on L^d targets when

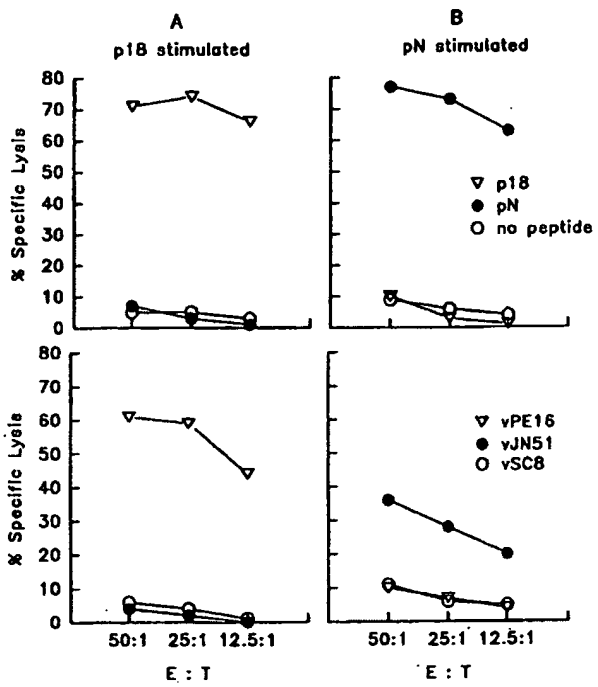

FIG. 3. Cytolytic activity of CTL from mice immunized with the Vac recombinant expressing chimeric epitope tan38. Polyclonal CTL derived from mice immunized with vtan38 were restimulated in vitro on either peptide p18 (A) or pN318-335 (B) and tested for recognition of exogenous (upper panels) and endogenous (lower panels) antigens. J774.1 target cells were coated with 1 μM peptide p18 or pN318-335 for 15 min at 37°C prior to the addition of effectors or infected with Vac recombinants expressing gp160 (vPE16), a truncated JN protein (vJN51), or a heterologous protein (vSC8). E:T, effector/target cell ratio.

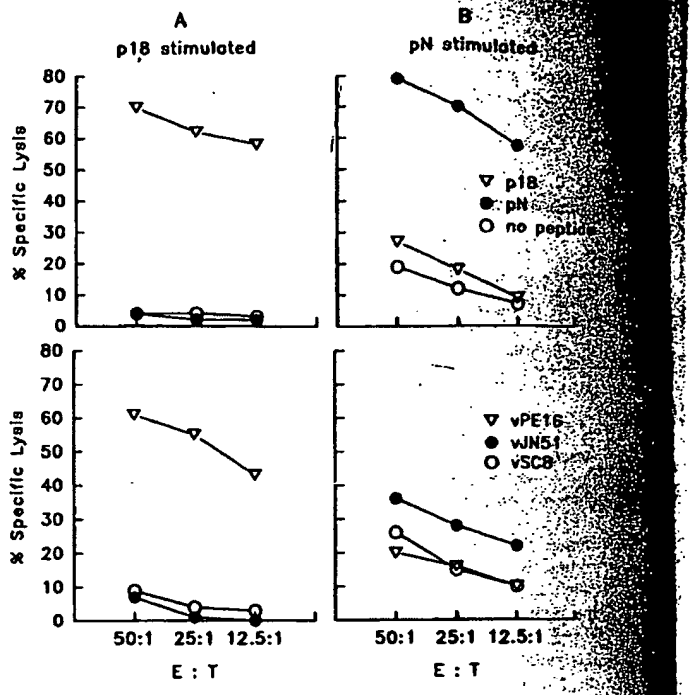

FIG. 4. Cytolytic activity of CTL from mice immunized with Vac recombinants expressing chimeric epitope tan2. Polyclonal CTL derived from mice immunized with vtan2 were restimulated in vitro on either peptide p18 (A) or pN318-335 (B) and tested for recognition of exogenous (upper panels) and endogenous (lower panels) antigens on J774.1 target cells as described in the legend to Fig. 3. E:T, effector/target cell ratio.

derived from vtan7 or vtan2. As the N-terminal sequences of the pN epitope were identical in all constructs (Fig. 1), reduced recognition can be attributed to either hindering C-terminal flanking residues introduced by the proximity of pN to p18 in the tan2 and tan7 chimeras or shorter overall peptide length. Reduced stability of the shorter peptides, comprising 31 (vtan2) and 36 (vtan7) aa, is unlikely to account for the inefficient presentation because the p18 epitope is presented efficiently in the identical targets (Fig. 2A and B). In addition, no differences in recognition were observed for a variety of JN protein truncations containing the pN epitope (2).

Efficient recognition is an insufficient criterion to ensure in vivo induction, as demonstrated by the failure of a minigene-encoded CTL epitope to induce antigen-specific CTL, despite its ability to sensitize targets (15). vtan38, which was efficient at sensitizing targets for lysis by both gp160- and JN-specific CTL (Fig. 2), was tested for the ability to prime secondary in vitro JN- and gp160-specific CTL. Spleen cells from vtan38-immunized mice were divided into three cultures and stimulated with either p18, pN318-335, or both peptides. Effectors from each group were tested for recognition of J774.1 targets infected with Vac recombinants expressing either gp160 (vPE16) or a truncated JN protein (vJN51; 2) and targets coated with either peptide. vtan38-induced CTL, restimulated with the individual peptides, were specific for the respective native antigens and corresponding peptides (Fig. 3A and B). Lysis of pN peptide-coated targets was consistently higher than that of targets sensitized with the endogenous JN epitope, suggesting that the endogenous concentration of the pN epitope is limiting (Fig. 3B). vtan38-induced effector cells stimulated with both peptides recognized targets expressing both native antigens but not targets infected with a heterologous Vac recombinant (data not shown). No differences were detected between the activity of CTL derived from vtan38-immunized mice and that of CTL from mice immunized simultaneously with Vac recombinants expressing gp160 aa 281 to 348 (v18-76; 3) and JN aa 301 to 351 (vJN51; 2) individually (data not shown). Furthermore, prior studies demonstrated that antigen-specific CTL were not primed in vitro by peptide stimulation (data not shown). Therefore, expression of both epitopes from a single gene was as effective as expression from the separate minigenes for CTL induction and the presence of two immunodominant epitopes did not inhibit CTL priming to either epitope.

JN-specific CTL recognized both $H\text{-}2^d$ and L^d targets infected with vtan2 weakly compared with vtan38-infected cells (Fig. 2). Therefore, vtan2 was tested for priming of both gp160- and JN-specific CTL. Effector cells were divided and restimulated in vitro with either peptide and tested for lysis of targets expressing the individual antigens or coated with peptide (Fig. 4). As expected, the cytolytic activity of p18-restimulated CTL (Fig. 4A) was comparable to that of vPE16-induced CTL (Fig. 2). No recognition of pN-coated targets was detected. The activity of pN-restimulated effectors (Fig. 4B) was similar to that of CTL from vtan38 (Fig. 3B) and coimmunized mice. As noted above, the L^d-restricted response for the cytosolic JN epitope was consistently lower than the D^d-restricted response

despite efficient recognition of peptide-coated cells. Although vtan2 was less efficient than vtan38 at sensitizing targets for lysis by JN-specific CTL (Fig. 2), no significant differences in the CTL activities of in vitro-restimulated spleen cells from vtan2-, vtan7 (data not shown)-, or vtan38 (Fig. 3)-immunized mice were observed. These results suggest that despite differential recognition of individual epitopes in vitro, the linkage of distinct epitopes restricted to different class I molecules can induce broadly reactive CTL within a single haplotype. Weak recognition in vitro does not necessarily correlate with a lack of CTL induction in vivo, consistent with results obtained by analysis of positional effects on class I presentation of a murine cytomegalovirus epitope (7). However, CTL activity from secondary cultures is the most sensitive CTL detection system (4, 23) and often does not reflect biologically relevant levels of CTL memory required for a protective response (4, 7, 23). Comparison of the threshhold required for recognition in vitro versus CTL induction in vivo is not feasible, as the local MHC-peptide complex concentration on the surface of virus-infected tissues in vivo is difficult to estimate. In addition, CTL induction in vivo requires accessory adhesion molecules, which may significantly lower the threshhold of MHC-peptide complexes required for induction (14, 24). In contrast to our findings, failure to induce CTL despite highly efficient recognition in vitro has also been described (15). These discrepancies between antigenicity and immunogenicity may reflect additional requirements for $CD4^+$ T-helper cells and/or the engagement of different subsets of antigen-presenting cells by the respective immunizing agent.

Flanking sequences may be critical in class I antigen presentation by hindering proteolysis and/or transport of peptides into the endoplasmic reticulum and thereby limiting the number of potential antigenic class I epitopes (6, 8, 19, 22). The proximal location of the heterologous N-terminal MHV epitope did not interfere with presentation of the C-terminal HIV epitope, confirming that altered N-terminal flanking sequences and the absence of C-terminal flanking residues have no effect on D^d-restricted presentation in vitro and CTL induction in vivo. Conversely, C-terminal sequences flanking the N-terminal MHV epitope exerted a negative effect on the efficiency of presentation. Additional analysis of a chimeric peptide containing the epitopes in reverse order with a single-residue spacer, namely, the HIV epitope in the N-terminal position and the MHV epitope in the C-terminal position (MRGPGRAFVTIEAPTAGAFFF), demonstrated highly efficient recognition of the MHV epitope and very poor recognition of the HIV epitope, confirming preferential presentation of the C-terminal epitope. Similar to other constructs, CTL induction to both epitopes was detected by using secondary stimulated cultures (data not shown). Consistent with previous reports, these results imply that processing of the C-terminal flanking residues is more selective than events at the N terminus (8, 13). This study supports the relevance of flanking sequences for optimal presentation of proximal epitopes. The induction of CTL specific for both native MHV N protein and HIV gp160 by chimeric peptides containing as little as one or two intervening residues supports the feasibility of a multi-T-cell epitope minigene approach to providing broad protective immunity. Direct correlation between in vitro efficiency of recognition and in vivo CTL induction requires CTL frequency analysis. Potential formation of novel hybrid epitopes, documented for class II peptides (20), was not detected. This approach offers the advantage of selectively inducing heterogeneous CTL to epitopes from different proteins of a single pathogen and to epitopes from distinct virus strains, thereby minimizing escape via antigenic drift. A single report demonstrates the efficacy of this approach in conferring protective immunity to lymphocytic choriomeningitis virus by immunization with chimeric peptides comprising linked epitopes restricted to MHC molecules of different haplotypes (27). Our data show that this strategy can be extended to include immunodominant epitopes presented by different MHC molecules within one haplotype.

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Silencing of Immunodominant Epitopes by Contiguous Sequences in Complex Synthetic Peptides[1]

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We have previously shown that the T cell response to the synthetic peptide cI_{12-26}:$NP_{365-380}$ (covalently linked epitopes of λ repressor (cI) and influenza A nucleoprotein (NP) polypeptides) requires amino acid sequences located in the junctional region between the cI_{12-26} and $NP_{365-380}$ epitopes in the H-2^d and H-2^k haplotypes. In this study, we show that the dominant epitope of cI_{12-26}:$NP_{365-380}$ in H-2^b mice is also located within the junctional region of the peptide, indicating that the same amino acid sequence is immunodominant in three different H-2 haplotypes. Based on results using fixed APC, there was no qualitative difference in epitope recognition due to antigen processing. In addition, antigen presentation by APC expressing mutant I-A molecules constructed by hemiexon shuffling of regions of the molecule containing primarily β sheet or α helix showed that many different substitutions were permissive for at least one of the T hybridomas. More importantly, however, when the junctional sequences are covalently linked in composite synthetic peptides containing additional previously defined T cell epitopes, antigenicity of the immunodominant junctional region was silenced and a new epitope assumed immunodominance. Thus, immunodominance does not correlate with the primary amino acid sequence of the potential epitope. Instead, the immunodominant epitope is determined by complex interactions among the epitopes, which most likely depend on the structural conformation of the composite peptide.

INTRODUCTION

The T cell response to most complex protein antigens is typically limited to a few, and frequently a single, immunodominant epitope (1). There is substantial evidence that a number of different mechanisms operating at different stages of an immune response can determine the immunodominance of a specific epitope (2, 3). First, processing by the antigen presenting cell can determine immunodominance. In this case, specific epitopes can be preferentially selected by the order of protein unfolding

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and denaturation (and hence the kinetics of availability of particular processed peptides) and by the sites of proteolytic cleavage, which can either select or destroy a specific potential epitope (4, 5). Second, the affinity of processed peptides for an MHC molecule can determine immunodominance at the level of competition for binding to the antigen binding site, both *in vivo* and *in vitro* (6, 7). Third, a defect in the T cell receptor repertoire, due to a "hole" in the repertoire, can cause the failure to recognize a specific epitope which has been successfully processed and presented (8, 9). In addition, recent work from our laboratory has shown that the T cell receptor repertoire can positively select one immunodominant epitope from two potential epitopes even though both epitopes are presented by APC and there is not a hole in the TCR repertoire (10).

Most studies of immunodominance have attempted to dissect T cell responses to complex protein antigens. In contrast, our approach has been to link covalently multiple previously defined and well-studied short T cell epitopes to produce increasingly complex composite synthetic peptides. In previous studies, we investigated recognition of the cI_{12-26}:$NP_{365-380}$ peptide,[3] which is composed of amino acids 12–26 of λ repressor protein cI linked to amino acids 365–380 of nucleoprotein of influenza A/NT/60/68. These studies demonstrated two mechanisms which contributed to the determination of immunodominance. First, intramolecular competition between the covalently linked T cell epitopes occurred at the level of presentation, and second, intermolecular competition between binding to different MHC molecules (I-A versus I-E) can determine immunodominance. For example, in H-2^d mice, immunodominance is determined by intramolecular competition between two distinct epitopes located on the cI_{12-26}:$NP_{365-380}$ peptide (11); in H-2^k mice, immunodominance is determined by intermolecular competition between epitopes of cI_{12-26}:$NP_{365-380}$ binding to the I-A or the I-E MHC molecules (12).

Based on the previous work showing that the junctional region of the cI_{12-26}:$NP_{365-380}$ peptide is required for immunodominance in both H-2^d and H-2^k mice, in this study we have investigated the potential dominance of this peptide in H-2^b mice, which normally are nonresponders to the cI_{12-26} and the $NP_{365-380}$ epitopes. Our current results show that the junctional sequence of cI_{12-26}:$NP_{365-380}$ is also immunodominant in the H-2^b haplotype; however, the identical sequence is antigenically silent in the context of another complex peptide, CS:cI_{12-26}:$NP_{365-380}$, composed of three previously described epitopes including CS derived from the circumsporozoite coat protein of falciparum malaria. Furthermore, the immunodominant epitope of CS:cI_{12-26}:$NP_{365-380}$ is located within the CS:cI_{12-26} junction (the cI_{12-26}:$NP_{365-380}$ and CS epitopes are silent). However, the CS:cI_{12-26} junctional epitope is not dominant following immunization with the CS:cI_{12-26} peptide. Thus, in our system the interaction between multiple potential epitopes, rather than the primary sequence of a specific epitope, determines immunodominance.

METHODS

Animals. Strain C57BL/6 mice 8 to 12 weeks old were obtained from the Jackson Laboratory (Bar Harbor, ME).

Peptides. The amino acid sequence of peptides used in these studies is depicted in Fig. 1. All peptides were synthesized by the solid-phase method of Merrifield (13) with

[3] Abbreviations used: cI, λ repressor protein; NP, nucleoprotein; CS, circumsporozite coat protein; OVA, ovalbumin.

PEPTIDE	SEQUENCE
cl_{12-26}	LEDARRLKAIYEKKK
$NP_{365-380}$	IASNENMDAMESSTLE
CS	NANPNANPNANP
$CS_{\Delta 2}$:cl_{12-26}	NANPLEDARRLKAIYEKKK
$CS_{\Delta 1}$:cl_{12-26}	NANPNANPLEDARRLKAIYEKKK
CS:cl_{12-26}	NANPNANPNANPLEDARRLKAIYEKKK
CS:cl_{12-26}:$NP_{365-380}$	NANPNANPNANPLEDARRLKAIYEKKKIASNENMDAMESSTLE
cl_{12-26}:$NP_{365-380}$	LEDARRLKAIYEKKKIASNENMDAMESSTLE
J_{3-22}	DARRLKAIYEKKKIASNENM
J_{6-22}	RLKAIYEKKKIASNENM
J_{9-22}	AIYEKKKIASNENM
J_{9-25}	AIYEKKKIASNENMDAM
J_{9-28}	AIYEKKKIASNENMDAMESS
J_{11-28}	YEKKKIASNENMDAMESS
J_{13-28}	KKKIASNENMDAMESS
J_{15-28}	KIASNENMDAMESS
cl_{12-26}:P:$NP_{365-380}$	LEDARRLKAIYEKKKPIASNENMDAMESSTLE
NP:P:cl_{12-26}	IASNENMDAMESSTLEPLEDARRLKAIYEKKK
OVA:cl_{12-26}	SQAVHAAHAEINEAGRLEDARRLKAIYEKKK
Eβ:cl_{12-26}	LENKRAEVDTVCRLEDARRLKAIYEKKK
CS:$NP_{365-380}$	NANPNANPNANPIASNENMDAMESSTLE

FIG. 1. Amino acid sequences of synthetic peptides are depicted by the single-letter amino acid code, and all peptides were produced by a continuous synthesis. cl_{12-26} corresponds to residues 12 to 26 of the λ repressor cI. $NP_{365-380}$ corresponds to residues 365 to 380 of the influenza nucleoprotein. cl_{12-26}:$NP_{365-380}$ is a synthetic chimeric peptide whose sequence consists of cl_{12-26} (amino end) followed by $NP_{365-380}$. Entries of the form "J_{x-y}" refer to segments within the 31-amino-acid peptide cl_{12-26}:$NP_{365-380}$. cl_{12-26}:P:$NP_{365-380}$ has the same sequence as cl_{12-26}:$NP_{365-380}$, with the exception of an additional proline residue inserted between the cl_{12-26} and $NP_{365-380}$ halves. In $NP_{365-380}$:P:cl_{12-26} the cl_{12-26} and $NP_{365-380}$ moieties were exchanged, still separated by a proline residue. CS refers to three repeats of $NANP_{365-380}$ derived from the circumsporozoite coat protein of *falciparium malaria*. CS:cl_{12-26}:$NP_{365-380}$ includes the CS, cl_{12-26}, and $NP_{365-380}$ epitopes. OVA:cl_{12-26}, Eβ:cl_{12-26}, and CS:cl_{12-26} are composite peptides formed by linking amino acids 324–339 of ovalbulmin, the third hypervariable region of I-Eβ^d, and CS with cl_{12-26}, respectively; CS:$NP_{365-380}$ includes the CS and $NP_{365-380}$ epitopes.

an Applied Biosystems 430A peptide synthesizer. *t*-Butoxylcarbonyl amino acids were coupled to hydroxymethylphenylacetoaminomethylpolystyrene resins, and the peptide assemblies were carried out by using *t*-butoxylcarbonyl amino acids (Peninsula Laboratories, Belmont, CA) to produce 0.5 mmol of each peptide. The resins were treated with anhydrous hydrogen fluoride in the presence of 1 ml of *p*-cresol and 1 g of *p*-thiocresol for 1 hr at 0°C, and were extracted with diethyl ether followed by 30% glacial acetic acid. The peptides were desalted on a Sephadex G-25 column (2.5 × 90 cm) equilibrated with 4 *M* glacial acetic acid. The amino acid composition of the resin-linked peptides and the desalted peptides corresponded to the expected compositions. Selected peptides were subjected to protein sequence analysis using an Applied Biosystems 470A protein sequencer. Several peptides were also isolated by high-pressure liquid column chromatography on a Vydac C4 column eluted with 0.1% trifluoroacetic acid and 0 to 60% acetonitrile gradient (1 hr). The purity of the peptides analyzed as determined by protein sequence analysis and/or high-pressure liquid column chromatography is 90 to 95%.

Cell lines. BW5147$\alpha\beta^-$ was a gift of Dr. W. Born (14). HT-2 was a gift from Dr. D. Raulet (15). TA3 (I-$A^{d/k}$, I-$E^{d/k}$) was a gift of Dr. L. Glimcher (16). Class II major

histocompatibility complex L cell transfectants NABB.1F (bbb bbb), FT2.3H (bbb bbb), FT5.7H (bbb kkk), FT3.4H (kkk bbb), NA13B.1H (bbb kbb), NT18.11A (kbb kbb), NA13K.1H (kkk kbb), NA14B.1H (bbb bkb), NA5B.1H (bbb bdb), RT8.12H (ddd bbb), NA26B.2H (bbb dbb), RT 2.3.3H (ddd ddd), and RT4.15HP (kkk kkk) were a generous gift from Drs. R. Germain and N. Braunstein (17). The six-letter code depicts the haplotype of the three segments of the α and β chain segments, respectively. The first and second segments include amino acids 1–38 and 39–65 of Aα, and 1–49 and 50–96 of Aβ, respectively. Cell surface I-A expression was determined as described (18). All L cell transfectants were maintained in 15 μg/ml hypoxanthine, 0.2 μg/ml aminopterin, and 5 μg/ml thymidine, except NA26B.2H and NT18.11A which were maintained in 6 μg/ml mycophenolic acid, 250 μg/ml xanthine, and 15 μg/ml hypoxanthine, until 1 day prior to use. The 3A9 T hybridoma was a gift of Dr. Paul Allen (19). All cultures and assays were performed in RPMI with 10% FCS (GIBCO, Grand Island, NY), 10 m*M* glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml), and 2×10^{-5} *M* 2-mercaptoethanol.

Isolation of T cell hybridomas. Mice were immunized subcutaneously with 50 μg of peptide in 200 μl of 50% complete Freund's adjuvant (GIBCO). Seven to ten days later, popliteal, paraaortic, and inguinal lymph nodes were removed and cultured according to the method of Kappler and Marrack (20). A single cell suspension was prepared by pressing nodes through a fine wire mesh. The cell suspension, approximately 10^8 cells, was rinsed three times, and then incubated in 75-cm^2 flasks at a concentration of 4×10^6 cells/ml with a 10 μM concentration of the immunogen peptide. After 2 days, viable cells were isolated by fractionation with Ficoll–Metrizoate. A cell suspension of equal numbers of viable lymphocytes and BW$\alpha\beta^-$ cells was prepared and rinsed three times in RPMI. The cell suspension was centrifuged at 800*g* for 10 min, the supernatant was removed, and 1 ml of polyethylene glycol (Boehringer-Mannheim Biochemicals, Indianapolis, IN) was added gently to the pellet. The reaction mixture was incubated at 37°C, and 0.5, 1, 2, and 4 ml of RPMI were added to it after 1, 2, 4, and 8 min had elapsed, respectively. The cell suspension was then brought to a volume of 40 ml by adding enriched medium which included 50% supernatant from M12 cells, 20% FCS, 30% RPMI, with 10 m*M* glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml), and 2×10^{-5} *M* 2-mercaptoethanol. After incubating at 37°C for at least 2 hr, the cell suspension was brought to a volume of 80 ml, and plated onto 96-well flat-bottomed plates with 100 μl/well. One day later 50 μl of 3× hypoxanthine and azaserine (final concentration 1 μg/ml azaserine and 100 μM hypoxanthine) was added to each well. Hybridomas appeared in the wells 7 to 21 days later and were expanded to near confluence in 24-well plates, and then 100 μl of resuspended confluent cultures from 2 ml wells was plated with 5×10^4 NABB.1F APC plus 10 μm peptide in a final volume of 200 μl in 96-well flat-bottomed plates. After 24 hr, 50 μl of supernatants was harvested and tested for IL-2.

Lymph node proliferation assay. Briefly, lymphocytes were prepared from draining lymph nodes of mice immunized subcutaneously with 100 μg peptide in CFA. The cells were then plated in a half-area 96-well plate (Costar, Cambridge, MA) at 4×10^5 cells per well with conditioned media or serial dilutions of peptide in a final volume of 100 μl. After 48 hr, 1 μCi [^{3}H]thymidine in 50 μl was added to each well. The cells were harvested 8 to 12 hr later, and thymidine incorporation was determined as described (21).

IL-2 colorimetric tetrazolium assay. Aliquots of 5×10^4 T hybridomas were added to 5×10^4 APC per well plus indicated concentration of peptide in final volume of 200 μl. Supernatants were harvested after 24 hr and assayed for IL-2 content. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO) was dissolved in PBS at 5 mg/ml and filtered. Fifty microliters of supernatant was incubated for 20–24 hr with 10^4 HT-2 in 100 μl of cells in microtiter plates (15). Ten microliters of stock MTT solution was added per well and incubated at 37°C for 4 hr. One hundred microliters of 0.04 *N* HCl in isopropanol was added to all wells. Optical density was determined at 570 nm with a Model 450 microplate reader (Bio-Rad, Richmond, CA) (22).

APC fixing. For fixed-cell dose–response assays, L cell transfectants were plated in 96-well flat-bottomed plates and grown to confluence. The supernatant medium was removed, and the cells were washed twice with 200 μl per well of sterile PBS. Fifty microliters of PBS with 0.05% glutaraldehyde (Sigma) was added to each well and removed after 60 sec. To quench residual glutaraldehyde, 200 μl of 0.25 *M* HCl–lysine in PBS was added to each well, and the cells were washed twice in PBS, as before. Aliquots of conditioned media, hybridomas, and serial dilutions of peptide were added to a final volume of 200 μl per well, and, after incubation, supernatants were collected and assayed for IL-2 content, as described. Bacteriophage repressor cI protein amino-terminal fragment (residues 1–102) was prepared as previously described (23) and used as positive control for the fixing of the APC. Inhibition was greater than 90% in all experiments.

RESULTS

Previous work by our laboratory has shown that the cI_{12-26}:$NP_{365-380}$ peptide contains an immunodominant epitope in both the H-2^d and H-2^k haplotypes (11, 12). The cI_{12-26} (I-A^d and I-E^k restricted) and $NP_{365-380}$ (I-A^k restricted) peptides were previously identified as dominant epitopes in response to the cI or NP proteins (21, 23). However, the sequences recognized by responding T cells following immunization with the cI_{12-26}:$NP_{365-380}$ peptide in both cases are not contained within either the cI_{12-26} or the $NP_{365-380}$ portion of the composite peptide; rather, the immunodominant epitope requires residues from the carboxy portion of cI_{12-26} as well as residues from the $NP_{365-380}$ moiety. It has previously been shown that H-2^b mice fail to recognize either the cI_{12-26} or the $NP_{365-380}$ epitopes (21, 24). Therefore, we asked if the junctional sequences which produced immunodominant responses in both the H-2^d and H-2^k haplotypes would also be immunogenic in H-2^b strain mice. C57BL/6 mice were immunized with the cI_{12-26}:$NP_{365-380}$ peptide in CFA and the proliferative response of lymph node cells was determined (Fig. 2). The results show a strong proliferative response to cI_{12-26}:$NP_{365-380}$ whereas there was no detectable response to either the cI_{12-26} or $NP_{365-380}$ or the negative control CS.peptides.

Characterization of T hybridomas. To analyze peptide recognition at the clonal level, T cell hybridomas were produced from C57BL/6 mice immunized with cI_{12-26}:$NP_{365-380}$ (Table 1). A total of 305 hybridomas produced in two separate fusions were tested for responses to the cI_{12-26}, $NP_{365-380}$, and cI_{12-26}:$NP_{365-380}$ peptides. Seventy-five of the T hybridomas recognized only the cI_{12-26}:$NP_{365-380}$ peptide, whereas two hybridomas recognized cI_{12-26} and nine hybridomas recognized $NP_{365-380}$. All of the hybridomas which recognized either cI_{12-26} or $NP_{365-380}$ also recognized the cI_{12-26}:

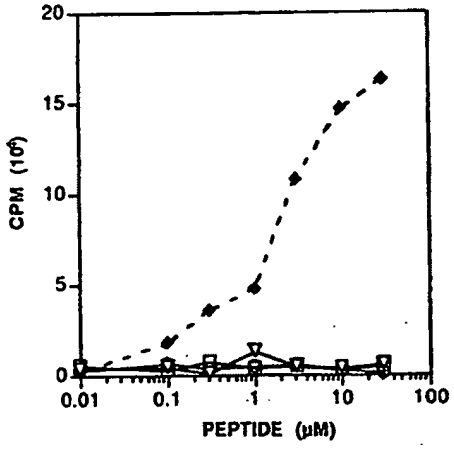

FIG. 2. C57BL/6 mice were immunized with 50 μg of cI_{12-26}:$NP_{365-380}$ subcutaneously in CFA. Seven days later, LN cells were removed and stimulated with cI_{12-26}:$NP_{365-380}$ (♦), cI_{12-26} (O), $NP_{365-380}$ (□), and CS (▽) and proliferation was determined by [^{3}H]thymidine incorporation as described. Results are representative of three separate experiments. In all figures, values plotted at the origin represent 0 μ*M* peptide, and the parallel slash located on the *x* axis represents a discontinuous break in the logarithmic scale. In all figures symbols are drawn relative to the *x* and *y* axis.

$NP_{365-380}$ peptide. Thus, the overall pattern of recognition by the T hybridomas in which 87% of the hybridomas recognize only the cI_{12-26}:$NP_{365-380}$ peptide correlates with the results of the lymph node proliferation.

Fine specificity of epitope recognition by T hybridomas. To determine the fine specificity of the T cell recognition of the cI_{12-26}:$NP_{365-380}$ peptide, individual hybridomas were tested against a panel of analogue peptides. This panel included progressive amino- and carboxy-terminal deletions, insertion of a proline residue between the cI_{12-26} and $NP_{365-380}$ moieties, reversal of the cI_{12-26} and $NP_{365-380}$ sequences separated by a proline residue, and the substitution of the cI_{12-26} sequence with unrelated epitopes including CS, OVA, and Eβ (peptides shown in Fig. 1). The response of a representative T hybridoma 58-21 depicts the recognition of several analogue peptides including J_{3-22},

TABLE 1

Specificity of T Cell Hybridomas

	Hybrids tested	cI_{12-26}:$NP_{365-380}$ responders	cI_{12-26} responders	$NP_{365-380}$ responders
Fusion 1	146	45	2	9
Fusion 2	159	30	0	0
Total	305	75	2	9

Note. T cell hybridomas were produced from C57BL/6 mice immunized with cI_{12-26}:$NP_{365-380}$ and tested for IL-2 secretion in response to peptides cI_{12-26}, $NP_{365-380}$, and cI_{12-26}:$NP_{365-380}$. Positive wells were determined by a minimum stimulation index >6. IL-2 was determined as described by [^{3}H]thymidine incorporation by the HT-2 indicator cell line.

J_{6-22}, J_{9-25}, and J_{9-28} as well as the cI_{12-26}:$NP_{365-380}$ peptide (Fig. 3a). The results from similar analysis of 16 different T hybridomas showed that 14 (88%) recognized at least one of the junction peptides (not shown). Although the fine specificity varied among the hybridomas, they all recognized peptides within the region spanning the 6th to 25th residue. In addition, 15 of the 16 T hybridomas did not recognize the cI_{12-26}:P:$NP_{365-380}$ peptide, and none of the hybridomas recognized the $NP_{365-380}$:P:cI_{12-26} peptide. Taken together, these results suggest that the panel of T hybridomas recognize an epitope located at the junction between the cI_{12-26} and $NP_{365-380}$ moieties.

Effects of antigen processing on peptide recognition. To evaluate the effects of antigen processing on the recognition of the dominant junctional epitope, APC were fixed by

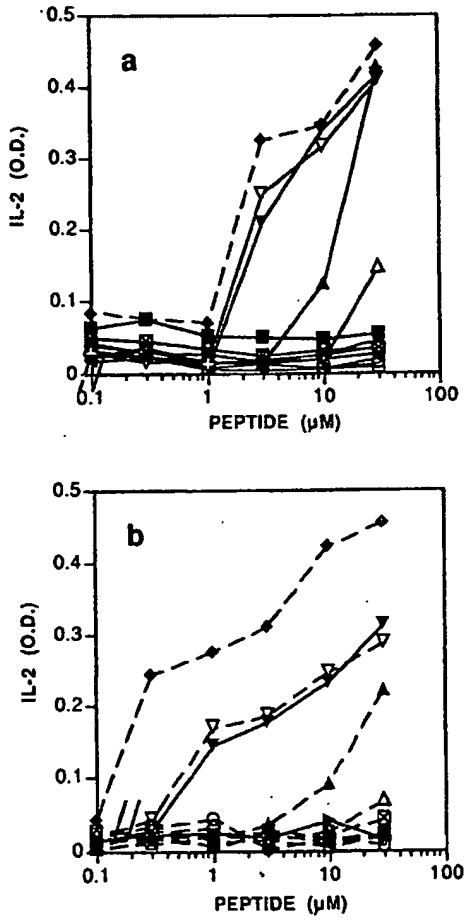

FIG. 3. Recognition of junctional peptides with live (a) and fixed (b) APC. T hybridoma 58-21 was stimulated using NABB.1F APC. Responses to peptides cI_{12-26}:$NP_{365-380}$ (◆), J_{3-22} (▽), J_{6-22} (▼) J_{9-25} (△), and J_{9-28} (▲). There was no detectable response to peptides cI_{12-26} (○), $NP_{365-380}$ (□), J_{9-22} (◀), J_{11-28} (▶), J_{13-28} (✣), J_{15-28} (✱), cI_{12-26}:P:$NP_{365-380}$) (■), CS:$NP_{365-380}$ (●), and $NP_{365-380}$:P:cI_{12-26} (⊠). IL-2 secretion was determined as described. Results are representative of three separate experiments.

crosslinking with glutaraldehyde and tested for the ability to stimulate the T hybridomas. As shown in Fig. 3b, the hybridoma 58-21 recognizes the same subset of peptides with fixed compared to live APC. Similar analysis of four additional T hybridomas also failed to detect qualitative differences between live and fixed APC (not shown). However, some quantitative differences of the relative potency of the stimulatory peptides is observed after fixation of the APC. In particular, the relative potency of the J_{3-22} and J_{6-22} peptides is decreased relative to cI_{12-26}:$NP_{365-380}$ when presented by fixed APC. These results suggest that although antigen processing is not required for recognition, it can alter the antigenicity of the peptides recognized by the 58-21 hybridoma.

Structural requirements of the I-A molecule for T cell recognition of cI_{12-26}:$NP_{365-380}$. The previous experiments focused on delineating the contribution of the various peptide sequences in determining immunodominance of a particular epitope. To investigate the contribution of the I-A^b MHC molecule to the recognition of the cI_{12-26}:$NP_{365-380}$ peptide, a panel of APC expressing mutant I-A molecules was used to present cI_{12-26}:$NP_{365-380}$ to the T hybridomas. The mutant I-A molecules were formed by switching hemiexons derived from the various H-2 haplotypes (25). Based on the Brown model, segment one contains residues of the β sheet and segment two contains residues of the α helix (see Methods) (26). A representative experiment showing the response by five T hybridomas to the cI_{12-26}:$NP_{365-380}$ peptide presented by the NA13B.1H APC is depicted in Fig. 4. The NA13B.1H APC express a mutant β sheet of the I-A β chain which is derived from the H-2^k sequence, with the remaining regions of both the α and β chains derived from the native I-A^b sequences. Clearly two of the T hybridomas, 58-21 and 16-5, recognize cI_{12-26}:$NP_{365-380}$ presented by the mutant I-A molecule.

Similar analyses of the same five T hybridomas shown in Fig. 4 were performed with the complete panel of APC and the results are summarized in Table 2. These data show that six of the transfected APC expressing mutant I-A molecules can present

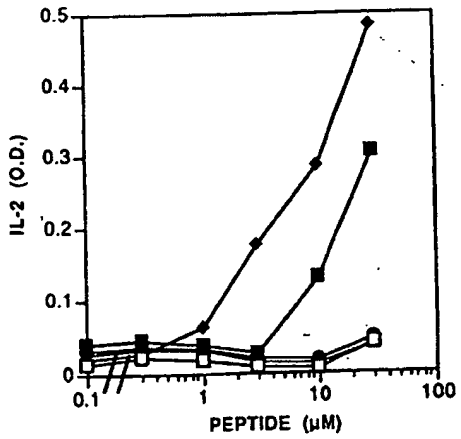

FIG. 4. Differential recognition of recombinant I-A molecules. T cell hybridoma 58-21 (■), 70-36 (●), 96-49 (▲), 16-5 (♦), and B6-6 (□) was tested for reactivity to NA13B.1H APC which expressed recombinant I-A molecules (bbb kbb), which has the b → k substitution of the β sheet in the I-A β chain. IL-2 was determined as described. Results are representative of two separate experiments.

TABLE 2

Responses to Exon-Shuffled I-A Molecules

APC	I-A		T hybridoma				
	α	β	58-21	70-36	96-49	16-5	B6-6
NABB.1F	bbb	bbb	0.320	0.366	0.376	0.334	—
FT5.7H	bbb	kkk	—	—	—	—	—
FT3.4H	kkk	bbb	—	—	0.370	—	—
NA13B.1H	bbb	kbb	0.304	—	—	0.482	—
NT18.11A	kbb	kbb	—	—	0.241	0.398	—
NA13K.1H	kkk	kbb	—	—	—	—	—
NA14B.1H	bbb	bkb	0.092	0.063	—	—	—
RT8.12H	ddd	bbb	0.078	—	0.501	—	—
NA26B.2H	bbb	dbb	—	0.092	—	—	—
NA5B	bbb	bdb	—	—	—	—	—
RT2.3.3H	ddd	ddd	—	—	—	—	—
RT4.15HP	kkk	kkk	—	—	—	—	—

Note. T hybridomas demonstrate different patterns of reactivity to APC expressing recombinant I-A molecules. B6-6 is a control T hybridoma specific for cI73-88 restricted by I-A^b. Each T cell hybridoma was tested as described.

cI_{12-26}:$NP_{365-380}$ to at least one of the hybridomas; however, none of the T hybridomas produced identical patterns of response to the panel of APC. It is remarkable that changes in either the α helix or the β sheet are permissive for some hybridomas. For example, hybridoma 70-36 recognizes cI_{12-26}:$NP_{365-380}$ in the context of both ab → k substitution of the α helix (NA14B.1H) and ab → d substitution of the β sheet (NA26B.2H) of the I-A β chain. Furthermore, the mutation of the β sheets of both the α and β chains with b → k substitutions (NT18.11A) is permissive for the T hybridomas 96-49 and 16-5. Also, a complete substitution of the α chain of b → k (FT3.4H) is permissive for hybridoma 96-49 and that of b → d (RT8.12H) is permissive for hybridomas 96-49 and 58-21.

Silencing of immunodominant epitopes in the context of complex synthetic peptides. Based on our current analysis of the cI_{12-26}:$NP_{365-380}$ peptide in the H-2^b haplotype plus our previous results in the H-2^d and H-2^k haplotypes showing the generation of an immunodominant epitope in all three haplotypes requiring portions of both the cI_{12-26} and $NP_{365-380}$ sequences, it would be tempting to speculate that the amino acid sequence located within the junctional region of the cI_{12-26}:$NP_{365-380}$ composite peptide is highly antigenic because it contains a structural motif with conserved elements immunodominant in multiple H-2 haplotypes. Such a motif could be termed a "super" epitope. To test this hypothesis, an additional peptide, CS:cI_{12-26}:$NP_{365-380}$, composed of three sequential epitopes was synthesized. This peptide contains 12 amino acids derived from the epitope of the coat protein of falciparum malaria linked to the cI_{12-26}:$NP_{365-380}$ sequence. Previous work established that the CS sequence, which includes three tandem repeats of Asn–Ala–Asn–Pro derived from the coat protein of falciparum malaria, was an immunodominant epitope of the coat protein in H-2^b strain mice (27). C57BL/6 mice were immunized with CS:cI_{12-26}:$NP_{365-380}$ and lymph node proliferation was determined to a panel of peptides representing the various

es

ybridoma

96-49	16-5	B6-6
0.376	0.334	—
—	—	—
0.370	—	—
—	0.482	—
0.241	0.398	—
—	—	—
—	—	—
0.501	—	—
—	—	—
—	—	—
—	—	—
—	—	—

PC expressing recombinant I-A
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the coat protein in H-2^b
$_{2-26}$:$NP_{365-380}$ and lymph
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previously defined epitopes (Fig. 5). The immunodominant response was not focused on the cI_{12-26}:$NP_{365-380}$ sequence or any of the individual epitopes including CS, cI_{12-26}, or $NP_{365-380}$. Rather, the immunodominant response was entirely contained within the CS:cI_{12-26} sequence. Thus, in the context of the CS:cI_{12-26}:$NP_{365-380}$ peptide, the J_{6-25} sequence shown to be immunodominant in the context of the cI_{12-26}:$NP_{365-380}$ peptide is essentially silent.

It was previously established that C57BL/6 mice were nonresponders to the cI_{12-26} epitope (24), whereas CS, as previously discussed, is an immunodominant epitope in this strain. To delineate the interaction between the CS and cI_{12-26} epitopes, C57BL/6 mice were immunized with CS:cI_{12-26} and lymph node proliferation was measured (Fig. 6). The results show that the CS peptide stimulates a strong proliferative response, although not as strong as the response to the immunogen CS:cI_{12-26}. Furthermore, because the response to the CS:$NP_{365-380}$ peptide is approximately equivalent to the CS:cI_{12-26} response (and both are approximately 1 order of magnitude greater than the response to CS), and because there is no obvious sequence homology between the carboxy moiety of the CS:cI_{12-26} and CS:$NP_{365-380}$ peptides, the enhanced response to these peptides may be due to lengthening the peptide at the carboxy end. Also, there was a modest response to the $CS_{\Delta 1}$ peptide, which has a deletion of one of the four amino acid repeats, whereas there was no detectable response to $CS_{\Delta 2}$, which has a deletion of two of the repeats. Most importantly, H-2^b mice respond to the CS epitope differently in the context of the CS:cI_{12-26}:$NP_{365-380}$ or the CS:cI_{12-26} peptides. Similarly, the response to the junctional epitope differs dramatically in the context of the cI_{12-26}:$NP_{365-380}$ or the CS:cI_{12-26}:$NP_{365-380}$ peptides.

DISCUSSION

Instead of dissecting the T cell response to a complex protein antigen, our approach has been to start with previously well-characterized T cell epitopes and to construct

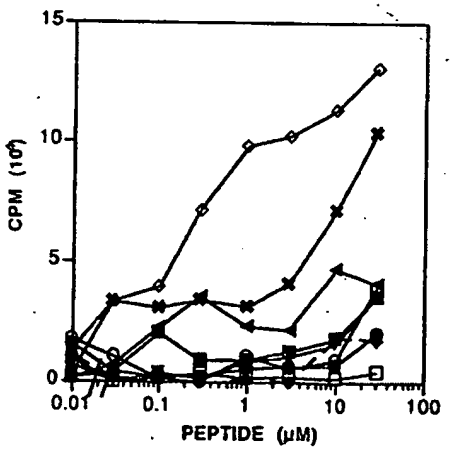

FIG. 5. C57BL/6 mice were immunized with 50 μg of CS:cI_{12-26}:$NP_{365-380}$ subcutaneously in CFA. Seven days later, lymph node cells were removed and stimulated with CS:cI_{12-26}:$NP_{365-380}$ (◇), cI_{12-26}:$NP_{365-380}$ (◆), CS:cI_{12-26} (✱), CS:$NP_{365-380}$ (■), cI_{12-26}:CS (◀), cI_{12-26} (○), $NP_{365-380}$ (□), and CS (●), and proliferation was determined by [^{3}H]thymidine incorporation as described. Results are representative of three separate experiments.

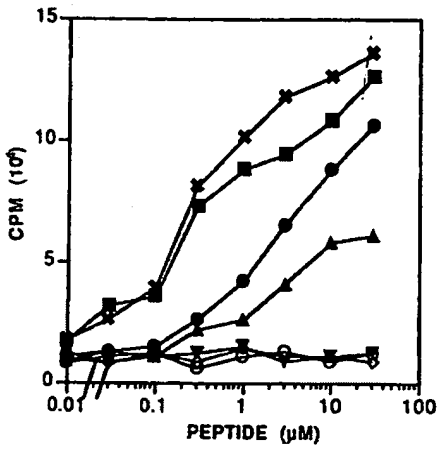

FIG. 6. C57BL/6 mice were immunized with 50 μg of CS:cI_{12-26} subcutaneously in CFA. Seven days later, LN cells were removed and stimulated with CS:cI_{12-26} (✱), $CS_{\Delta1}$:cI_{12-26} (◇), $CS_{\Delta2}$:cI_{12-26} (▲), cI_{12-26} (○), $NP_{365-380}$ (□), CS:$NP_{365-380}$ (■), and CS (●), and proliferation was determined by [^{3}H]thymidine incorporation as described. Results are representative of two separate experiments.

increasingly complex antigens by covalently linking multiple epitopes in a single synthetic peptide. In this study we investigated the T cell response of H-2^b C57BL/6 mice to the composite peptide cI_{12-26}:$NP_{365-380}$, which links the cI_{12-26} epitope of λ repressor with the $NP_{365-380}$ epitope of nucleoprotein. Previous studies established that C57BL/6 mice are nonresponders to the cI_{12-26} (24) and the $NP_{365-380}$ (21) epitopes. However, these data show that C57BL/6 mice do respond to the cI_{12-26}:$NP_{365-380}$ peptide based on both lymph node proliferation and the characterization of T cell hybridomas from two separate fusions. A detailed analysis of the fine specificity of 16 of the T hybridomas showed that they all recognized shorter peptides spanning the junctional region of cI_{12-26}:$NP_{365-380}$. For example, hybridoma 58-21 recognized peptides J_{3-22}, J_{6-22}, J_{9-25}, and J_{9-28}. Furthermore, 15 of 16 T hybridomas did not recognize the cI_{12-26}:$NP_{365-380}$ peptide, which has a single proline residue inserted between the cI_{12-26} and $NP_{365-380}$ sequences. In addition, the recognition of the T hybridomas was not qualitatively altered by fixing the APC, indicating that antigen processing was not required for recognition of the new epitope, although it does quantitatively alter the response by some hybridomas to specific peptides. The simplest interpretation of these results is that a new epitope, located within the junctional region of cI_{12-26}:$NP_{365-380}$, becomes immunodominant in H-2^b mice.

Previous groups have shown that short flanking sequences added to either the carboxy or the amino terminus can either enhance (28) or diminish (29) the response to the core region of an epitope. The response of hybridoma 58-21 shows that additions to either the carboxy or the amino terminus can enhance recognition of the core sequence. It is remarkable that the consensus sequence present in all of the junctional peptides recognized by 58-21 is J_{9-22}, but there is no detectable response by 58-21 to the J_{9-22} peptide. However, carboxy-terminal additions (J_{3-22} and J_{6-22}) or amino-terminal additions (J_{9-25} and J_{9-28}) restore antigenicity. Despite three separate immunizations of C57BL/6 mice, we could not detect significant lymph node proliferation; in addition, there was no detectable *in vitro* competition for presentation of the I-A^b-restricted

cI_{73-88} peptide. However, J_{9-22} was shown to be antigenic in both H-2^d and H-2^k mice (not shown). Therefore, these data are consistent with the hypothesis that J_{9-22} fails to bind I-A^b with significant affinity. Presumeably, either amino- or carboxy-terminal additions could both enhance peptide binding to I-A.

The determinant selection model of immunodominance proposes that the major factor controlling immunodominance is the ability of a peptide to bind to the antigen binding site of the MHC molecule. We directly investigated the contribution of the various domains of the I-A^b molecule by using APC transfected with hemiexon-shuffled I-A molecules to present cI_{12-26}:$NP_{365-380}$. Two conclusions are apparent from these experiments: first, each T hybridoma recognized the cI_{12-26}:$NP_{365-380}$ peptide in the context of a different subset of the mutant I-A molecules, and second, many different substitutions were permissive for at least one of the T hybridomas. In particular, permissive substitutions of the first hemiexon (containing primarily the β sheet region) included at least one example of the b, d, and k sequences for both the I-A α and β chains. Similarly, permissive substitutions of the second hemiexon (containing primarily the α helix region) of the I-A α chain also included b, d, and k and those of the I-A β chain included b and k. In fact, the only substitution that was not recognized by a T hybridoma in at least one of the mutant APC was a d haplotype α helix of the I-A β chain. This functional degeneracy is all the more remarkable because it required a total of only four T hybridomas. Thus, there is considerable degeneracy and/or redundancy in the TCR–peptide–MHC interaction in our system.

These results differ substantially from the results of Lechler *et al.* studying T cell recognition of antigen in the context of hemiexon-shuffled I-A molecules (25). In their study, only 2 of 21 T hybridomas recognized antigen in the context of a mutant I-A molecule, and in each case only a single mutant I-A molecule was permissive; in addition, substitution of the β sheet of the β chain eliminated recognition by 90% of the antigen specific responses. Our system differs in the fact that the cI_{12-26}:$NP_{365-380}$ peptide is known to be immunogenic in the H-$2^{b,d}$ and H-2^k haplotypes. Therefore, cI_{12-26}:$NP_{365-380}$ clearly binds I-A molecules of all three haplotypes, which could at least partially explain the degeneracy. However, the fine specificity analysis of cI_{12-26}:$NP_{365-380}$ recognition in the b, d, and k haplotypes suggests that there may be subtle differences in binding among the three I-A molecules. More importantly, degeneracy of binding would still not account for the additional degeneracy involving TCR contact residues of the α helix. For example, T hybridoma 96-49 tolerates both the k and d substitutions of the α helix of the α chain (compatible with predominant recognition of polymorphic residues of the β chain). More remarkably, hybridoma 58-21 tolerates the α helix substitutions of k haplotype in the β chain and d haplotype in the α chain. None of the hybridomas tested recognized cI_{12-26}:$NP_{365-380}$ presented by APC expressing allogeneic I-A^d or I-A^k.

The simplest explanation of these data is that the peptide–MHC binding and the TCR recognition of the peptide–MHC complex involve multiple noncovalent interactions. This explanation is compatible with a model in which the immunodominant response to this highly immunogenic peptide involves relatively high-affinity peptide–MHC interactions. Thus, if one of multiple peptide–MHC binding interactions is eliminated with the mutant I-A molecule, recognition by the T hybridoma may be quantitatively reduced but not abolished. In addition, many of the residues predicted to be involved in Ag binding and T cell contact are conserved among the I-$A^{b,d}$ and I-A^k molecules; therefore, these residues could contribute to recognition in the mutant

I-A molecules. Due to the different levels of surface MHC expression between different APC transfected with I-A, it is not possible to compare quantitatively the response of the T hybridoma to cI_{12-26}:$NP_{365-380}$ presented by the various mutant APC. In addition, we cannot exclude the possibility that substitutions in one region of the molecule could induce conformational changes in another region. However, as described under Methods, I-A expression has been determined by flow cytometry analysis with mAb, and every APC has stimulated at least one T hybridoma in previous analysis (not shown).

It is remarkable that the junctional sequences of cI_{12-26}:$NP_{365-380}$ form an immunodominant epitope in three different H-2 haplotypes (b, d, and k). One hypothesis of these observations is that immunodominance of the cI_{12-26}:$NP_{365-380}$ sequence is due simply to a consensus motif of immunodominance which is functional in multiple haplotypes. However, the T cell response by C57BL/6 mice to the CS:cI_{12-26}:$NP_{365-380}$ peptide, which contains the complete cI_{12-26}:$NP_{365-380}$ sequence, clearly disproves this hypothesis. The immunodominant determinant following CS:cI_{12-26}:$NP_{365-380}$ immunization is contained within the CS:cI_{12-26} region, and the cI_{12-26}:$NP_{365-380}$ epitope is silenced. Previous reports established that CS was an immunodominant epitope of the falciparum malaria coat protein in H-2^b mice (27). However, there was no significant response to either CS or the previously discussed junctional epitope of cI_{12-26}:$NP_{365-380}$. These results indicate that the complex interaction among multiple potential epitopes, rather than the primary sequence of a single epitope, determines immunodominance in our system. This conclusion is further supported by the response to the CS:cI_{12-26} peptide. In this case the dominant response is focused on the CS epitope, which was immunologically silent in the context of the CS:cI_{12-26}:$NP_{365-380}$ peptide.

In conclusion, the major factors determining the immunodominance of various epitopes linked in complex synthetic peptides composed of multiple previously well-defined T cell epitopes are not the innate characteristics or primary sequences of a given epitope but rather the complex interaction among the epitopes in the context of the composite peptide. Although not directly addressed by our studies, these effects are most likely due to different structural conformations created by linking multiple epitopes in the composite peptides. Thus, the results in our system are consistent with a model, previously proposed by Sercarz and co-workers, in which antigen binds to the MHC molecule prior to the completion of unfolding and proteolytic cleavage; therefore, certain potential epitopes become masked (cryptic) while other epitopes which are exposed earlier during processing are preferentially presented (30). Alternatively, different conformations could have different binding affinities for MHC molecules. Thus, potentially immunodominant epitopes can be silenced by contiguous but distant changes in the polypeptide. Because peptide vaccines may require the incorporation of multiple epitopes, further understanding of the interactions among potential epitopes in the context of complex polypeptides may be important for the design of successful vaccines as well as understanding T cell recognition of complex protein antigens.

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IMMUNODOMINANCE: INTRAMOLECULAR COMPETITION BETWEEN T CELL EPITOPES

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We have used an approach of linking previously characterized T cell epitopes into immunologically complex synthetic peptides in order to investigate the mechanism of immunodominance. Our results show that first, cI_{12-26} is highly dominant following immunization with the lambda repressor (cI) protein, but is a minor epitope in the context of the cI:NP peptide. In contrast, the dominant epitope in response to the cI:NP peptide is a new junctional epitope, which is composed of sequences derived from both the cI and influenza nucleoprotein (NP) segments of the composite peptide. Second, T cell recognition of cI:NP is not significantly altered by Ag processing, based on results from glutaraldehyde-fixed APC. Third, the relative affinities of cI and cI:NP for MHC binding are similar, based on in vitro competition, excluding competition at the level of MHC binding as the determinant of immunodominance. Taken together, these results are consistent with the hypothesis that immunodominance of cI:NP is determined by peptide conformation, which affects the configuration of peptide binding to MHC, thus altering T cell recognition. In conclusion, immunodominance is not simply a function of the primary amino acid sequence, but is a function of the context of the epitope within the protein molecule.

The immune response of T cells after immunization with complex antigens is commonly focused on a small number of major epitopes, and in many cases a single immunodominant epitope (1–9). In the case of BALB/c mice immunized with cI,[2] more than 95% of the maximal lymph node T cell proliferative response is stimulated by peptides spanning amino acids 12 to 26 (cI_{12-26}) (9). The phenomenon of T cells recognizing a limited number of dominant epitopes is observed in all MHC haplotypes. Previous work from our laboratory has shown that of a total of 13 different MHC haplotypes immunized with cI, 7 responded to only a single dominant epitope, and the largest number of epitopes identified in a single strain was 3 (10). Although T cell responses in all of the strains were limited to a few epitopes, the specific epitopes recognized varied among strains. The strain specificity presumably is dependent upon binding interactions between the processed peptides and the polymorphic MHC molecules.

Clearly, one requirement for immunodominance is binding of the peptide/processed Ag to the MHC molecule. Based on the model of MHC class II molecules proposed by Brown et al. (11), there is a single Ag binding site located at the amino terminus of the molecule. Therefore, competition between the various processed peptides for binding to the MHC molecule is one mechanism that could determine immunodominance (12). However, a number of peptides that bind to MHC molecules but do not correlate with immunogenicity have been identified. In a detailed analysis of peptides from staphylococcal nuclease, 30% of peptides that bound to MHC were not immunogenic (13). The authors concluded that "holes" in the TCR repertoire prevented recognition of these peptides. Similarly, although cI_{12-26} binds to both I-A^d and I-E^d, the T cell response is restricted overwhelmingly to I-A^d due to a hole in the TCR repertoire for cI_{12-26} presented by I-E^d (14). In contrast, several epitopes have been identified that do not cause T cell responses following immunization with the intact protein, but which produce significant responses following immunization with the synthetic peptide (15). For example, although more than 95% of the T cell response of BALB/c mice to cI is specific for cI_{12-26}, immunization with the peptide cI_{46-62} causes a marked lymph node proliferation (16). Thus, cI_{46-62} is a perfectly good immunogen, but is immunologically silent following immunization with cI. Presumably events prior to T cell recognition involving protein unfolding, Ag processing, or competition for binding to MHC prevent significant immunogenicity of cI_{46-62} after cI immunization.

To investigate the mechanisms of immunodominance, we have constructed synthetic composite peptides consisting of multiple previously identified T cell epitopes. The advantage of this approach is that epitopes previously well characterized can be covalently linked and functionally analyzed in the context of the new composite polypeptide. In particular, the immunogenicity of epitopes cI_{12-26} (amino acids 12–26 of cI) and NP_{335-80} (amino

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[2] Abbreviations used in this paper: cI, lambda repressor; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NP, influenza nucleoprotein.

acids 365–380 of NP) (17) was investigated in the context of the 31-residue composite peptide cI:NP. Our results indicate that immunodominance of cI:NP in BALB/c mice is not directed to the cI_{12-26} moiety but rather to a new epitope. In addition, immunodominance of major versus minor epitopes is determined by intramolecular competition between multiple overlapping epitopes present in the composite peptide.

MATERIALS AND METHODS

Animals. Strain BALB/c mice were obtained from The Jackson Laboratory (Bar Harbor, ME).

Peptides. All peptides were synthesized by the solid-phase method of Merrifield (18) with an Applied Biosystems (Foster City, CA) 430A peptide synthesizer. *t*-Butoxylcarbonyl amino acids were coupled to hydroxymethylphenylacetoaminomethylpolystyrene resins, and the peptide assemblies were carried out by using *t*-butoxylcarbonyl amino acids (Peninsula Laboratories, Belmont, CA) to produce 0.5 mmol of each peptide. The resins were treated with anhydrous hydrogen fluoride in the presence of 1 ml of *p*-cresol and 1 g of *p*-thiocresol for 1 h at 0°C, and were extracted with diethyl ether followed by 30% glacial acetic acid. The peptides were desalted on a Sephadex G-25 column (2.5 × 90 cm) equilibrated with 4 M glacial acetic acid. The amino acid composition of the resin-linked peptides and the desalted peptides corresponded to the expected compositions. Selected peptides were subjected to protein sequence analysis using an Applied Biosystems 470A protein sequencer. Several peptides were also isolated by high-pressure liquid column chromatography on a VYDAC (Hesperia, CA) C4 Column eluted with 0.1% trifluoroacetic acid and 0 to 60% acetonitrile gradient (1 h). The purity of the peptides analyzed as determined by protein sequence analysis and/or high-pressure liquid column chromatography is 90 to 95%.

Cell lines and culture. BW5147$\alpha\beta^-$ variant was a gift of Dr. W. Born (University of Colorado Health Sciences Center, Denver, CO). CTLL.2 was a gift from Dr. D. Raulet (University of California, Berkeley, CA). TA3 ($I\text{-}A^{d/k}$, $I\text{-}E^{d/k}$) was a gift of Dr. L. Glimcher (National Institutes of Health, Bethesda, MD). Class II MHC L cell transfectants RT 2.3.3H ($I\text{-}A^d$) and RT 10.3H2 ($I\text{-}E^d$) were a gift from Dr. R. Germain (National Institutes of Health, Bethesda, MD). All cultures and assays were performed in RPMI with 10% FCS (both from GIBCO, Grand Island, NY), 10 mM glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml), and 2×10^{-5} M 2-ME (complete medium). Plating of fusions and assays were performed in 96-well flat-bottomed plates (half-area wells for lymph-node proliferation assays, full-area wells otherwise). A mixture of azaserine (1 μg/ml) and hypoxanthine (100 μM) was used in hybrid selection.

Isolation of T cell hybridomas. Mice were immunized subcutaneously with 50 μg peptide in 200 μl of 50% complete Freund's adjuvant (GIBCO). Eight to ten days later, popliteal, paraaortic, and inguinal lymph nodes were removed and cultured according to the method of Kappler et al. (19). A single cell suspension was prepared by pressing nodes through a fine wire mesh. The cell suspension (approximately 10^6 cells) was rinsed three times in conditioned media, and then incubated in 75 cm^2 flasks at a concentration of 4×10^6 cells/ml with 10 μg/ml immunogen peptide. After 2 days, viable cells were isolated by fractionation with Ficoll-Metrizoate. A cell suspension of equal numbers of viable lymphocytes and fusion partner cells was prepared and rinsed three times in RPMI. The cell suspension was centrifuged at 800 × *g* for 10 min, the supernatant was removed, and 1 ml of polyethylene glycol (Boehringer Mannheim Biochemicals, Indianapolis, IN) was gently added to the pellet. The reaction mixture was incubated at 37°C, and 0.5 ml, 1 ml, 2 ml, and 4 ml of RPMI were added to it after 1 min, 2 min, 4 min, and 8 min had elapsed, respectively. The cell suspension was then brought to a volume of 40 ml by adding enriched medium including the supernatant of M12 cells incubated in conditioned media for 2 days (50%), FCS (20%), RPMI (30%), 10 mM glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml), and 2×10^{-5} M 2-ME. After incubating at 37°C for at least 2 h, the cell suspension was brought to a volume of 80 ml, and plated onto 96-well flat-bottomed plates with 100 μl/well. One day later 50 μl of 3× hypoxanthine and azaserine in enriched media was added to each well. Hybridomas appeared in the wells 7 to 21 days later. Hybridomas were expanded to near-confluence in 24-well plates, then 100 μl of resuspended confluent cultures from 2-ml wells were plated with 5×10^4 TA3 Ag presenting cells, and either conditioned media, cI_{12-26} (5 μg/ml), $NP_{365-380}$ (5 μg/ml), or cI:NP (10 μg/ml), in a final volume of 200 μl in 96-well flat-bottomed plates. Cultures were incubated for 24 h, and 50 μl of the supernatants were harvested.

IL-2 assay. Fifty microliters of supernatant were frozen for no more than 2 h at −70°C. Aliquots of 1×10^4 cells of CTLL.2 or HT-2, IL-2-dependent T cell lines were added. [^{3}H]Thymidine (1 μCi/well) was added 18 to 24 h later; the cells were harvested 4 to 8 h later with an automated cell harvester (Skatron Inc., Sterling, VA). Incorporated thymidine was detected by scintillation counting.

Dose-response assay. L cell transfectants RT 2.3.3H ($I\text{-}A^d$) or RT 10.3H2 ($I\text{-}E^d$) were plated in 96-well flat-bottomed plates and grown to confluence. Aliquots of 5×10^4 hybridomas were added to each well. The final volume was brought to 200 μl with conditioned media plus serial dilution of peptide. Supernatants were recovered and assayed for IL-2 content as described.

Lymphocyte proliferation assay. T lymphocytes were prepared from lymph nodes of mice primed with cI:NP as described. The lymphocytes were then plated on a half-area 96-well plate (Costar no. 3696, Cambridge, MA) at 4×10^5 cells/well with conditioned media or serial dilution of peptide in a final volume of 100 μl. Supernatants (50 μl/well) were removed 24 h later and assayed for IL-2 content as described. To measure lymphocyte proliferation, [^{3}H]thymidine (1 μCi) in 50 μl of conditioned media was added to each well after a 48-h incubation. The cells were harvested 8 to 12 h later, as described. Each assay has been repeated at least twice, with two to three mice each time. The titration curves of Ag in proliferation and IL-2 assays were comparable after we subtracted the background.

APC fixing. For fixed-cell dose-response assays, L cell transfectants RT 2.3.3H ($I\text{-}A^d$) or RT 10.3H2 ($I\text{-}E^d$) were plated in 96-well flat-bottomed plates and grown to confluence. The supernatant medium was removed, and the cells were washed twice with 200 μl/well of sterile PBS. Fifty microliters of PBS with 0.05% glutaraldehyde (Sigma Chemical Co., St. Louis, MO) were added to each well and removed after 80 s. To quench residual glutaraldehyde, 200 μl of 0.25 M HCl-lysine in PBS was added to each well, and the cells were washed twice in PBS, as before. Aliquots of conditioned media, hybridomas, and serial dilutions of peptide were added to a final volume of 200 μl/well, and, after incubation, supernatants were collected and assayed for IL-2 content, as described. Bacteriophage repressor cI protein amino-terminal fragment (residues 1–102) was used as positive control for the fixing and was prepared as described by Lai et al. (9).

Colorimetric tetrazolium assay. MTT (Sigma) was dissolved in PBS at 5 mg/ml and filtered. Supernatant (50 μl) was incubated for 20–24 h with 10^4 HT-2 cells in 100 μl in microtiter plates. Ten microliters of stock MTT solution were added per well and incubated at 37°C for 4 h. One hundred microliters per well of 0.04 N HCl in isopropanol were added. Optical density was determined at 570 nm with a model 450 Microplate Reader (Bio-Rad, Richmond, CA).

RESULTS

To investigate the relative immunodominance of covalently linked T-cell epitopes, we synthesized the peptide cI:NP (composed of cI_{12-26} plus $NP_{365-380}$; Fig. 1) and tested the T cell response to the peptide in BALB/c mice ($H\text{-}2^d$). Based on previous work, the cI_{12-26} moiety of the composite peptide is immunogenic in the $H\text{-}2^d$ haplotype, whereas $NP_{365-380}$ is not immunogenic in $H\text{-}2^d$ but is restricted by $I\text{-}A^k$ (and by D^b on class I-restricted T-cells) (20). We immunized BALB/c mice with the cI:NP peptide and assayed for in vitro lymph node cell proliferation in response to either the cI:NP peptide, cI_{12-26} alone, or $NP_{365-380}$ alone (Fig. 2). We found in three separate experiments that the response to the combination peptide was markedly stronger than that to cI_{12-26}, and that $NP_{365-380}$ produced no detectable response. A representative experiment shown in Figure 2 shows a maximal response of 17,800 cpm for cI:NP compared with 5480 cpm for cI_{12-26} (background equals 1890 cpm). Based on three separate experiments, the range of the stimulation index was 7.6–9.4 for cI:NP and 2.8–3.6 for cI_{12-26}. This result was unexpected, because previous work from our laboratory had shown that within the cI protein cI_{12-26} was the major epitope, constituting more than 95% of the total T cell response.

PEPTIDE	SEQUENCE
cI_{12-26}	LEDARRLKAIYEKKK
$NP_{365-380}$	IASNENMDAMESSTLE
cI:NP	LEDARRLKAIYEKKKIASNENMDAMESSTLE
J_{3-22}	DARRLKAIYEKKKIASNENM
J_{6-22}	RLKAIYEKKKIASNENM
J_{9-22}	AIYEKKKIASNENM
J_{9-25}	AIYEKKKIASNENMDAM
J_{9-28}	AIYEKKKIASNENMDAMESS
J_{11-28}	YEKKKIASNENMDAMESS
J_{13-28}	KKKIASNENMDAMESS
J_{15-28}	KIASNENMDAMESS
cI:P:NP	LEDARRLKAIYEKKKPIASNENMDAMESSTLE
NP:P:cI	IASNENMDAMESSTLEPLEDARRLKAIYEKKK
CS:NP	NANPNANPNANPIASNENMDAMESSTLE
CS:cI	NANPNANPNANPLEDARRLKAIYEKKK
CS:cI:NP	NANPNANPNANPLEDARRLKAIYEKKKIASNENMDAMESSTLE
CS	NANPNANPNANP

Figure 1. Amino acid sequences of synthetic peptides. cI_{12-26} corresponds to residues 12 to 26 of the cI. $NP_{365-380}$ corresponds to residues 365 to 380 of NP. cI:NP is a synthetic chimeric peptide whose sequence consists of cI_{12-26} (amino end) followed by $NP_{365-380}$. Entries of the form J_{xxx-xx} refer to segments within the 31-amino acid peptide cI:NP. cI:P:NP has the same sequence as cI:NP, with the exception of an additional proline residue inserted between the cI_{12-26} and $NP_{365-380}$ halves. In NP:P:cI the cI_{12-26} and $NP_{365-380}$ moieties were exchanged, still separated by a proline residue. CS refers to three repeats of NANP derived from the circumsporozoite coat protein of *falciparum malaria*. CS:cI, CS:cI:NP, and CS:NP are chimeric peptides formed by peptidically linking CS and cI_{12-26}; CS, cI_{12-26}, and $NP_{365-380}$; and CS and $NP_{365-380}$, respectively.

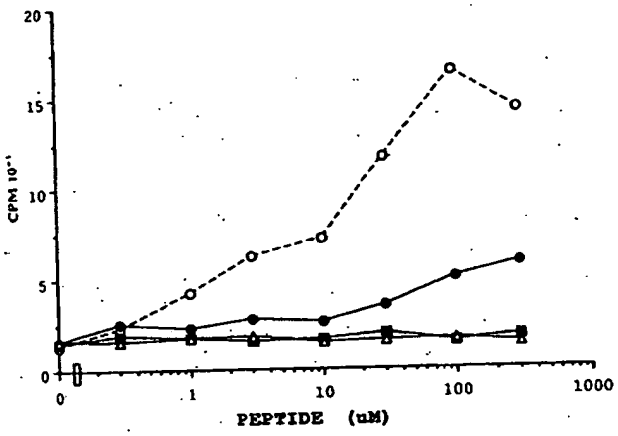

Figure 2. BALB/c lymph node proliferation analysis. Mice were immunized with 50 μg cI:NP subcutaneously in complete Freund's adjuvant. Seven days later, lymphocytes were removed and stimulated with cI:NP (○), cI_{12-26} (●), $NP_{365-380}$ (■), and $OVA_{324-339}$ (Δ), and proliferation was determined by [^{3}H]thymidine incorporation as described. In all figures, values plotted on the *y*-axis represent 0 μM peptide, and the rectangle located on the *x*-axis represents a discontinous break in the logarthmic scale.

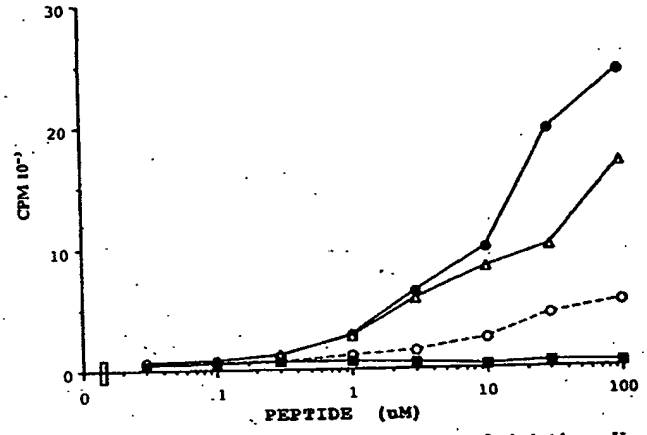

Figure 3. Weak response to cI:NP by a cI_{12-26}-specific hybridoma. Hybridoma 7B7.2, originally derived from a BALB/c mouse immunized with cI, was stimulated with peptides cI:NP (○), cI_{12-26} (●), $NP_{365-380}$ (■), and CS:cI (Δ), and IL-2 secretion was determined with CTLL indicator cells as described.

To determine if T cells specific for the cI_{12-26} epitope can recognize the composite peptide cI:NP, we tested the response of a panel of seven previously characterized T cell hybridomas specific for cI_{12-26} that were derived from BALB/c mice immunized with cI. Six of the hybrids (6C2, 7B7, 8F8.10, 9C12.7, 8F8.4, and 16H8.7) had a similar pattern of response, which is illustrated by the hybrid 7B7.2 (Fig. 3). In all of these hybrids the response to cI:NP was between 0.5 and 1.1 orders of magnitude lower than the response to cI_{12-26}. The single exception (15C9) responded to cI:NP slightly better than to cI_{12-26}. Hybridoma 15C9 uses a unique Vβ segment compared to the other six hybridomas (which all express Vβ8) (21), and produces a heteroclitic response to a His substitution of cI_{12-26} at position 22. More importantly, the decreased response to cI:NP was not due simply to the increased length of the composite peptide, because the response to another composite peptide CS:cI (composed of three NANP repeats derived from the circumsporzoite coat protein of *malaria falciparum* linked to cI_{12-26}; Fig. 1) was similar to the response to cI_{12-26} alone.

These results are compatible with several compatible hypotheses, including 1) cI:NP was binding to MHC in a new configuration that inhibited presentation of the cI_{12-26} epitope, 2) in the composite peptide the cI_{12-26} moiety adopts an altered conformation not recognizable by the cI_{12-26} specific T cells, and 3) Ag processing following immunization altered or destroyed the cI_{12-26} epitope in the composite peptide. To investigate these hypotheses, we generated T cell hybridomas from two separate fusions from BALB/c mice immunized with cI:NP. A total of 189 hybrids were screened, and 140 of these responded to test peptides. Among the responding hybrids, 110 (110/140 = 78.6%) responded only to cI:NP but not to cI_{12-26}; and 29 (29/140 = 20.7%) responded to both cI_{12-26} and cI:NP, while 1 (1/140 = 0.7%) responded only to cI_{12-26} (Table I). Of these hybrids, 149 were also tested for response against $NP_{365-380}$ without detection of reactivity. The increased proportion of hybridomas responding to cI:NP compared with cI_{12-26} correlates well with the

TABLE I
Specificity of T cell hybridomas[a]

	Hybrids Tested	cI:NP Responders	cI:NP and cI_{12-26} Responders	$NP_{365-380}$ Responders
Fusion 1	40	29	4	(Not tested)
Fusion 2	149	81	25	0
Total	189	110	29	0

[a] Hybridomas were tested for IL-2 secretion in response to peptides cI_{12-26}, $NP_{365-380}$, and cI:NP. Positive wells were determined by a stimulation index >3. IL-2 was determined as described by [^{3}H]thymidine incorporation by the CTLL indicator cell line.

increased magnitude of the responses to cI:NP in the LN proliferation assay of Figure 2, in which bulk T cell populations were used (Fig. 2).

The observation that 97% (29/30) of the hybrids that recognized cI_{12-26} also recognized cI:NP indicates that the cI_{12-26} epitope can be recognized within the composite peptide and that Ag processing of cI:NP does not quantitatively destroy presentation of the cI_{12-26} moiety. These results are consistent with the hypothesis that a new epitope, presumably overlapping the two original linked epitopes, is preferentially recognized in cI:NP. To delineate the putative new epitope, we selected nine T-cell hybrids for further analysis and stimulated them with a panel of overlapping peptides spanning the junctional region of cI:NP with serial amino- and carboxy-terminal deletions (Fig. 1). The sequences for two other peptides in this panel were generated by inserting a proline between the two original moieties of cI:NP, forming cI:P:NP, and by reversing the order of the two moieties in the second peptide while maintaining the junctional proline residue, forming NP:P:cI. The rationale for these modifications was twofold. First, prolines are known to drastically alter secondary protein structure, including the disruption of alpha helices, as well as potentiation of reverse turns (22). Therefore, the proline insertions may segregate the peptides into two immunogenic regions composed of the two original epitopes cI_{12-26} and $NP_{365-380}$, similar to domain segregation by the proline hinge region in Ig molecules (23). Second, the proline insertion changes the primary structure of the junctional region, which could disrupt T cell recognition or MHC binding of the putative new junctional epitope. We also included control variants CS:cI and CS:cI:NP, in which the amino termini were nonspecifically lengthened with the sequence NANP derived from the malaria coat protein.

The panel of T hybridomas was tested with both live and glutaraldehyde-fixed L cell APC transfected with I-A^d. Table II includes eight hybridomas specific for cI:NP and one that recognizes cI_{12-26}. The most striking observation is that three of the hybridomas do in fact recognize peptides that span the junctional region, in particular, in the region of amino acids 3–28. However, four additional hybridomas that also recognize cI:NP did not recognize any of the analog peptides tested. Although it is unlikely, we cannot exclude the possibility that these hybridomas might be specific for a processed peptide not included within our panel, or require a blocked α-NH_2 group at the amino terminus. Alternatively, these hybridomas may recognize a conformation of the junctional region present only in the longer cI:NP peptide. The latter hypothesis is supported by the observation that the hybridoma D2.157, which does recognize cI:NP but not NP or the junctional peptides, also recognizes CS:NP. The stimulatory peptides cI:NP and CS:NP share sequence identity only in the NP moiety without apparent homology in the amino half of the peptides. Thus, in this case, the conserved sequence between the stimulatory peptides are not sufficient for recognition, but additional nonhomologous sequences located in the amino-terminal region of the composite peptide are required. These nonhomologous sequences could alter MHC binding or directly alter the conformation of the NP moiety, in either case, favoring recognition by D2.157.

To examine the relative potency of the analog peptides, eight hybridomas were selected for fine specificity analysis. Figure 4 shows the results from two hybridomas, D2.164 and D2.219, both of which respond to junctional peptides spanning the region including residues 3–28. All of the hybridomas analyzed recognized cI:NP with a response equivalent to or stronger than their recognition of the junctional peptides. Other workers have shown that immunodominance of some epitopes in the response to protein antigens is controlled at the level of Ag processing (12, 15). To determine if Ag processing altered the response to our panel of peptides, we analyzed hybridoma responses with glutaraldehyde-fixed APC. The results from Figure 4 are summarized in Table III; they show that stimulatory peptides share the amino acid residues 9–22 of the composite peptide. Results from fixed APC are similar. As shown in Figure 5, although there are subtle quantitative differences in the response to J_{6-22} and J_{9-28}, the overall pattern of response is similar with live versus fixed APC. Our results from fixed APC support the hypothesis that immunodominance of the composite peptide cI:NP is not controlled at the level of Ag processing. Additional mechanisms include the possibility that the junctional region has a greater affinity for MHC binding than does cI_{12-26}, or that the conformation of the composite peptide affects the specificity of MHC binding or T cell recognition during Ag presentation. To evaluate the relative binding affinities of the various peptides for the I-A^d molecule, we performed a competition experiment using the DO.11.10 hybridoma specific for amino acids 324–339 of ovalbumin. In this experiment A20 APC were incubated with different concentrations of cI:NP, cI_{12-26}, $NP_{365-380}$, J_{3-22}, and J_{9-28} plus a fixed concentration of the $OVA_{324-339}$ peptide. The results show that the cI:NP and cI_{12-26} peptides specifically inhibit the ovalbumin response in a dose-dependent manner (Fig. 6). More importantly, both peptides inhibit the response with similar dose-response patterns, indicating that the cI:NP peptide does not have a significantly greater binding affinity for I-A^d compared with cI_{12-26}. In addition, significant competition by these peptides required a 1000 to 2000 M excess of competitor peptide, indicating that their affinity for I-A^d is low. Furthermore, the shorter junctional peptides did not demonstrate detectable competition, indicating that their relative affinities are significantly lower than that of cI:NP.

DISCUSSION

Our results indicate that immunodominance is not determined solely by the primary amino acid sequence of the epitope but is dependent upon the context of the epitope within a given polypeptide. In the context of the protein cI, the peptide cI_{12-26} is consistently immunodominant in H-2^d murine strains, contributing more than 95% of the total T cell response. It is notable that cI_{12-26}

	L	F	L	F	L	F	L	F	L	F	L	F	L	F	L	F	L	F
cI:NP	72	56	93	100	21	10	55	25	96	45	50	5	71	10	77	16	79	6
cI:P:NP	29	–	35	–	13	14	–	–	83	70	51	6	69	10	5	–	10	5
cI_{12-28}	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	10	10
$NP_{365-380}$	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
J_{9-28}	92	73	82	9	6	5	–	–	–	–	–	–	–	–	–	–	–	–
J_{9-25}	4	–	17	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
J_{9-22}	11	–	26	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
J_{6-22}	39	18	52	–	5	–	–	–	–	–	–	–	–	–	–	–	–	–
J_{3-22}	100	40	88	53	10	–	–	–	–	–	–	–	–	–	–	–	–	–
J_{11-28}	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
J_{13-28}	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
J_{15-28}	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
CS:NP	–	–	–	–	–	–	–	–	–	–	27	6	–	–	–	–	–	–
NP:P:cI	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

[a] Responses of hybridomas were determined against a panel of junctional peptides. The first number of each pair (L) corresponds to Ag presentation by live APC, the second (F) to presentation by fixed APCs. In both cases, the APC were I-A^d-transfected L cells. Results indicate IL-2 secretion in response to fixed concentration (5 μM) of synthetic peptides. Results are reported as percentages of the maximal response to cI:NP. IL-2 was determined as described.

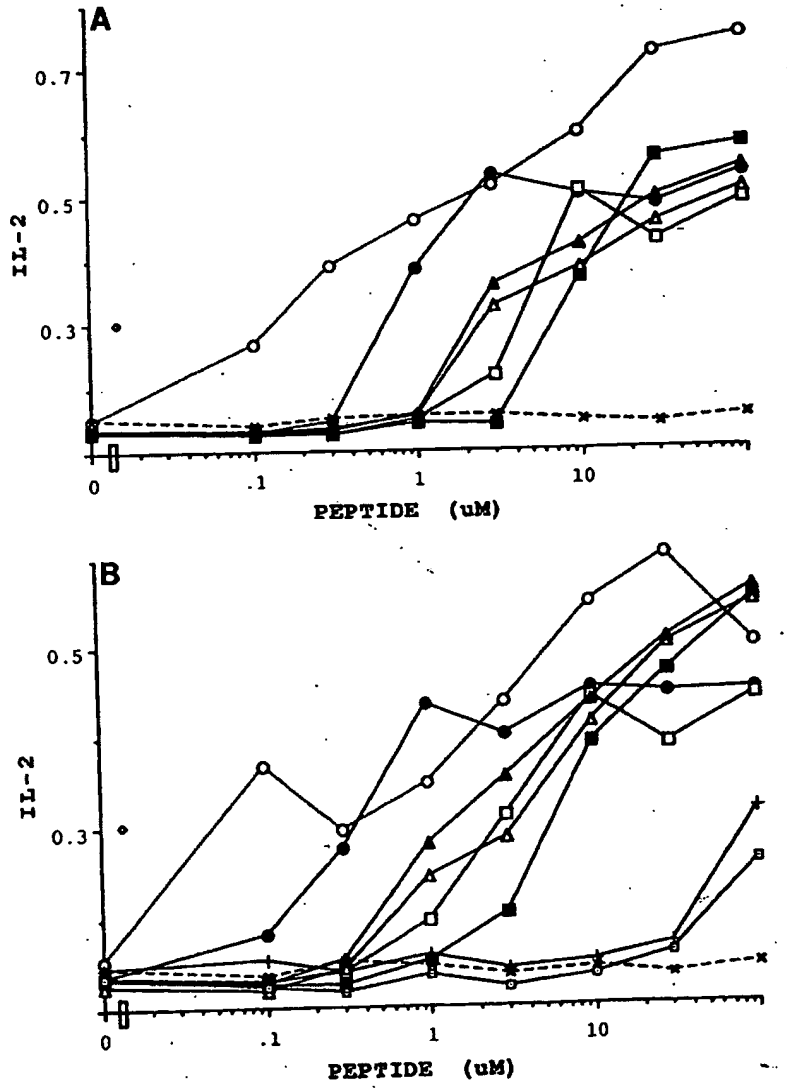

Figure 4. Recognition of junctional peptides with live APC. Hybridomas D2.164 (*a*) and D2.219 (*b*) were stimulated with peptides cI:NP (○), cI:P:NP (+), J_{3-22} (●), J_{6-22} (□), J_{9-22} (■), J_{9-25} (△), J_{9-28} (▲), and J_{11-28} (⊡) presented on live I-A^d-transfected L-cells. Peptides cI_{12-28}, $NP_{365-380}$, J_{13-28}, J_{15-28}, NP:P:cI, CS:NP all failed to stimulate either hybrid; the curve x – – – x represents the mean of negative responses. IL-2 was determined with the colorimetric tetrazolium assay as described.

is also a major epitope in the H-2^k and H-2^f MHC haplotypes.

To investigate the mechanisms of immunodominance of the cI_{12-28} epitope, we constructed polypeptides in which the cI_{12-26} epitope was covalently linked to other previously characterized epitopes, including $NP_{365-380}$, forming the composite peptide cI:NP. $NP_{365-380}$ was originally identified as a class I H-$2D^b$ restricted epitope of influenza A nucleoprotein (17), and subsequently $NP_{365-380}$ was also identified as a class II I-A^k restricted

[illegible]	LEDARRLKAIYEKKKIASNENMDAMESSTLE	0.3	0.1
J_{3-22}	DARRLKAIYEKKKIASNENM	0.9	0.2
J_{6-28}	AIYEKKKIASNENMDAMESS	3.0	1.2
J_{6-22}	RLKAIYEKKKIASNENM	6.4	2.5
J_{6-25}	AIYEKKKIASNENMDAM	8.2	3.3
J_{6-22}	AIYEKKKIASNENM	10.1	5.0
cl:P:NP	LEDARRLKAIYEKKKPIASNENMDAMESSTLE	–	80.4
J_{11-28}	YEKKKIASNENMDAMESS	–	>100
J_{13-28}	KKKIASNENMDAMESS	–	–
J_{15-28}	KIASNENMDAMESS	–	–
$NP_{365-380}$	IASNENMDAMESSTLE	–	–
NP:P:cl	IASNENMDAMESSTLEPLEDARRLKAIYEKKK	–	–
CS:NP	NANPNANPNANPIASNENMDAMESSTLE	–	–

[a] The responses by hybridomas D2.164 and D2.219 to a panel of analog peptides was determined at peptide concentrations from 0 to 100 μM. Results indicate the peptide concentration producing half-maximal IL-2 secretion compared with the cl:NP response. A value > 100 indicates a detectable, but less than half-maximal, response at 100 μM peptide. –, an undetectable response.

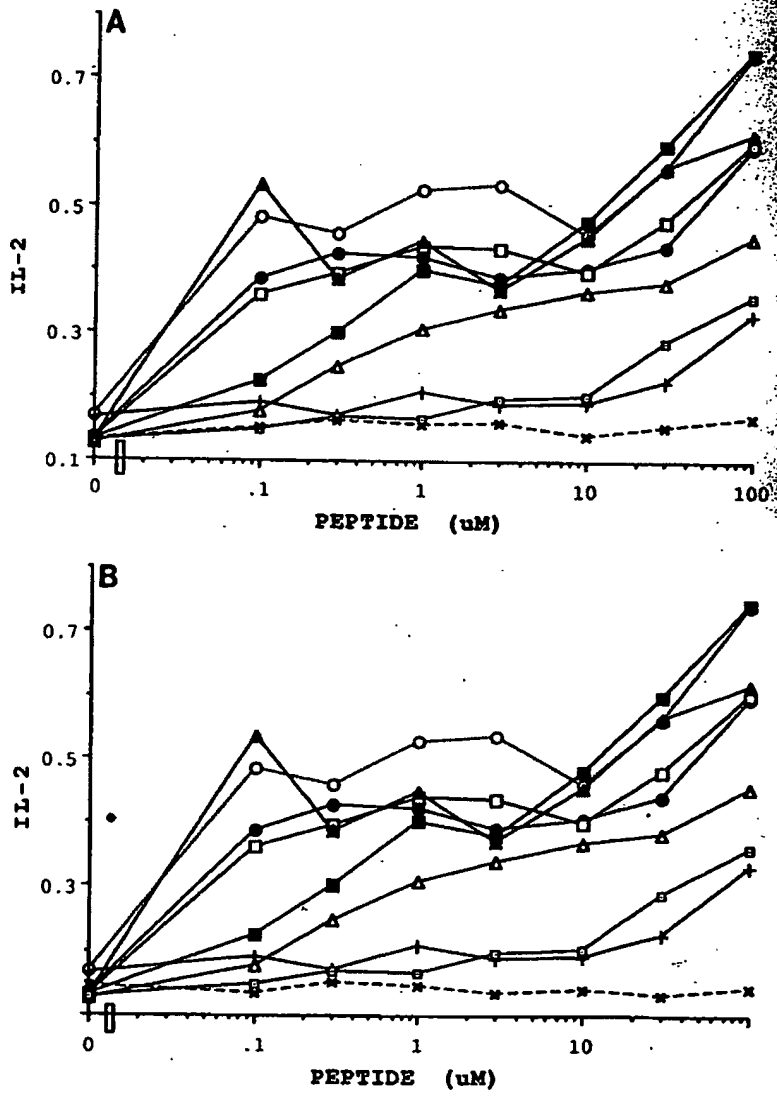

Figure 5. Recognition of junctional peptides with fixed APC. Hybridomas D2.164 (*a*) and D2.219 (*b*) were stimulated with peptides cl:NP (○), cl:P:NP (+), J_{3-22} (●), J_{6-22} (□), J_{9-22} (■), J_{9-25} (△), J_{9-28} (▲), and J_{11-28} (⊡) presented on fixed I-A^d-transfected L-cells. Peptides cl_{12-28}, $NP_{365-380}$, J_{13-28}, J_{15-28}, NP:P:cl, CS:NP all failed to stimulate either hybrid; the curve x – – – x represents the mean negative response. This assay was performed as described in Figure 4, except that after we grew the APCs to confluence in 96-well microtiter wells, they were fixed with glutaraldehyde before we added the hybridoma cells.

epitope for $CD4^+$ T cells, but was not immunogenic in the H-2^d haplotype (20). Following immunization of BALB/c mice with the composite cl:NP peptide, the potency of the cl_{12-26} epitope was dramatically inhibited. In contrast, six of seven T cell hybridomas tested specific for cl_{12-26} produced a reciprocal response pattern of weak recognition of the cl:NP peptide. Previous work had demonstrated that the six hybridomas had different patterns of fine specificity for the cl_{12-26} epitope, including differential recognition of cl_{12-24} versus cl_{14-26} and of His or Phe substitutions at position 22 (9). The fact that all six of these hybrids had weak recognition of cl:NP supports the

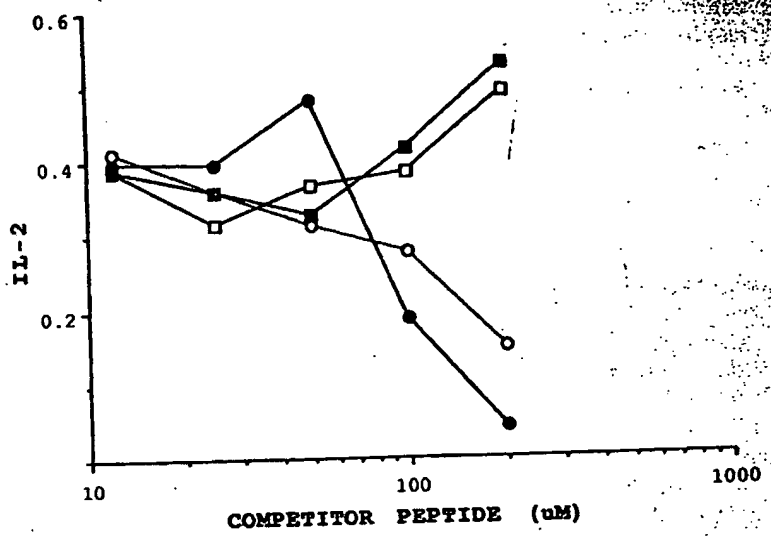

Figure 6. Competition by cI:NP peptides for Ag presentation. A20 APC were incubated with 1 μM $OVA_{324-339}$ peptide plus the indicated titrations of competitor peptides cI:NP (○), cI_{12-26} (●), $NP_{365-380}$ (□), J_{3-22}, J_{6-22}, J_{9-22}, and J_{9-28}; none of the junctional peptides showed detectable inhibition and are expressed as the mean (■). The APC were fixed with glutaraldehyde and thoroughly washed, and 5×10^4 DO.11.10 hybridoma cells/well were added for 24 h. IL-2 was determined by the colorimetric tetrazolium assay as described.

hypothesis that the cI:NP epitope presented in the context of I-A^d is substantially different from the cI_{12-26} epitope. The single exception, hybridoma 15C9, differs from the other six hybrids in two respects (9, 21). First, it expresses a non-Vβ8 TCR, whereas the other hybridomas all utilize Vβ8 genes. Second, it produces a heteroclitic response to the His substitution at position 22. The mechanism of the heteroclitic responses to the His analog and to cI:NP have not been determined. However, decreased immunogenicity is not a general property of polypeptides with multiple linked epitopes. Other experiments have shown that following immunization with another composite peptide CS:cI, the immunodominant epitope is not inhibited and is wholly contained within the cI_{12-26} moiety (unpublished observations).

Previous work by Gammon et al. (15), who used lysozyme as an Ag, has shown that the determination of immunodominance among multiple potentially immunogenic epitopes can be a function of Ag processing. The precise mechanisms have not yet been elicited but may include multiple steps of processing, including protein denaturation and unfolding, the sequence of epitope availability, the sites of proteolytic cleavage, or the affinity of binding to MHC molecules. Our results from fixed APC support the hypothesis that the various steps of Ag processing are not determining immunodominance in our system. Although it is less likely, we cannot exclude the possibility that in vivo processing preferentially selects the junctional epitope. However, because processing cannot be inhibited in vivo, we cannot directly test this possibility. Nevertheless, our in vitro results do show that processing is not required for T cell recognition of the junctional epitope. In addition, because 29/30 hybridomas specific for cI_{12-26} also recognized cI:NP in our initial testing, it is apparent that the cI epitope is not quantitatively destroyed by antigen processing and that functional quantities of the cI_{12-26} epitope are presented following stimulation with the cI:NP peptide. Recent work has shown that the in vivo half-life of peptides can be influenced by flanking sequences not required for immunogenicity; however, because cI_{12-26} is highly immunodominant following immunization with cI, our results cannot be explained by a short half-life of the cI_{12-26} peptide (24). The failure to recognize a specific epitope could also be due to a hole in the TCR repertoire. However, because previous data have shown that BALB/c mice respond to the cI_{12-26} epitope (9), it is apparent that the failure to generate a significant response to the cI moiety following cI:NP immunization is not due to a hole in the TCR repertoire.

Based on the fine specificity analysis of a panel of T cell hybridomas against analog peptides spanning the junctional region of cI:NP, it is clear that a major component of the dominant response is directed toward a junctional epitope represented by the peptides J_{3-22} through J_{9-28}. The simplest interpretation of these results is that cI:NP binds the I-A^d molecule in a configuration that exposes residues between 9 and 22 for T cell recognition due to the conformation of the composite peptide. Thus, it appears that the junctional epitope is shifted approximately nine amino acids relative to the cI_{12-26} moiety. Based on the predicted size of the MHC binding cleft, it is likely that different residues of the junctional peptides, compared with cI_{12-26}, contact the MHC molecule. In this case, cI:NP may also interact with sites, or binding pockets, in the MHC groove that are different from those bound by cI_{12-26}. Our results do not differentiate between the possibilities that peptide conformation prior to binding determines the contact site on the peptide for MHC binding, or that peptide conformation after binding affects T cell recognition. In either case, our results support the hypothesis that immunodominance of the junctional epitope of cI:NP is determined by intramolecular competition between the overlapping junctional and cI_{12-26} epitopes. Previous reports have shown that competition between different peptides for MHC binding, both in vitro and in vivo, can inhibit a potential response (25, 26). Our results extend these observations to the determination of immunodominance by intramolecular competition of overlapping epitopes.

The response pattern of the remaining hybridomas is also intriguing. These hybridomas respond to cI:NP but not to any of the panel of junctional peptides. The trivial hypothesis is that the panel of peptides did not include the precise analog recognized by these hybrids. However, previous work from several laboratories working with

altered conformation induced by the flanking sequences present in the longer composite peptide cI:NP.

Additional support for this hypothesis is the observation that two of these four hybridomas fail to recognize cI:P:NP with the proline insertion; whereas none of the other hybridomas tested were unresponsive to this peptide. The fact that these hybrids do not recognize any of the junction-spanning peptides, but that insertion of a proline into this region disrupts recognition, is consistent with a mechanism in which the proline dramatically alters the peptide conformation while bound within the putative Ag-binding groove of the MHC molecule during Ag presentation. Furthermore, the hybridoma D2.157, which recognizes cI:NP but none of the junction analogues, also recognizes CS:NP. The only observable sequence homology between these peptides is within the $NP_{365-380}$ moiety; however, these hybridomas do not recognize $NP_{365-380}$. These results are consistent with the hypothesis that the CS moieties induce a conformation change in the NP portion of the composite peptide, and the altered conformation is recognized by a different subset of T cells, presumably with a repertoire of TCR different from that utilized in CI_{12-26} recognition. A similar conformational change could occur in the cI:NP composite peptide.

Taken as a whole our results indicate that although the cI:NP composite peptide contains multiple T cell epitopes, it binds to $I\text{-}A^d$ in a configuration that presents a new junctional epitope for T cell recognition (and simultaneously inhibits presentation of the cI_{12-26} epitope, which was previously identified as an immunodominant epitope in this haplotype). The strongest evidence supporting this mechanism is the reciprocal patterns of response observed in the cI_{12-26} vs the cI:NP specific hybridomas—the former recognize the composite peptide approximately one order of magnitude more weakly than cI_{12-26}; whereas the latter recognize cI_{12-26} weakly or not at all. Presumably, cI:NP binds $I\text{-}A^d$ in one of two configurations, exposing either the junctional or the cI_{12-26} epitope, with the equilibrium strongly favoring presentation of the junctional epitope. This assumption is supported by the proportion of hybridomas recognizing cI:NP (79%) vs cI_{12-26} (21%) in our initial screening. In conclusion, immunodominance of the T cell response to the cI:NP peptide is determined by intramolecular competition between multiple overlapping, covalently linked T cell epitopes.

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Effect of epitope flanking residues on the presentation of N-terminal cytotoxic T lymphocyte epitopes

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We here demonstrate that placing two distinct influenza virus nucleoprotein epitopes at the N terminus of a cytosolic protein selectively blocks their presentation to specific cytotoxic T lymphocytes. The block is a cytosolic phenomenon, which can be overcome by distancing the epitope from the protein N terminus by two or more amino acids. Shortening the protein's C terminus fails to relieve the antigen presentation block. These results demonstrate that events at the N terminus of the target protein, rather than at its C terminus, are responsible for the lack of presentation of N-terminal epitopes. We also show that lack of presentation of N terminal epitopes is associated with a modification of the target protein which affects its electrophoretic mobility and isoelectric focusing point. This modification can be prevented by mutating the epitope's N-terminal flanking sequence, which results in an efficient presentation of the N-terminal epitope. Lack of presentation of the N-terminal epitopes results in a reduced ability of influenza-primed mice to clear acute infection with vaccinia virus encoding an N-terminal nucleoprotein epitope. Our results demonstrate that presentation of epitopes localized at the N terminus of cytosolic proteins can be modulated by events occurring at early stages of antigen processing.

Key words: Antigen presentation / MHC class I / Post-translation modification / Amino terminus

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1 Introduction

The majority of CTL recognize intracellular proteins in the form of peptides associated with MHC class I molecules. Newly synthesized proteins are degraded in the cytosol and peptides generated from them are then transported into the endoplasmic reticulum (ER) by the peptide transporter associated with antigen presentation (TAP) complex. In the lumen of the ER, peptides can be trimmed to their final size and are transported to the cell surface in association with MHC class I molecules [1].

The analysis of the events leading to antigen presentation demonstrate that several steps can influence the pool of antigenic peptides presented at the cell surface by MHC class I molecules. The very presence of peptide binding motifs indicates that MHC class I molecules play an important role in epitope selection. There is also growing evidence that TAP has some degree of peptide specificity [2, 3]. The cleavage specificity of cytosolic proteases for the generation of antigenic peptides is less well defined. Several lines of evidence suggest that a proportion of antigenic MHC class I epitopes are generated by the cytosolic protease proteasome (reviewed in [4]), though it is becoming clear that other proteases also play a role in this process [5–8]. The proteasome is a multisubunit complex with a central catalytic core of four stacked heptameric rings forming an enclosed cavity in which proteolysis takes place (reviewed in [9]). At least five proteolytic activities have been identified in this catalytic core with respect to amino acid residues after which cleavage can be found [10]. The efficiency of antigen processing by proteasomes appears to be governed in part by protein primary sequences and flanking CTL epitopes [11–15]. Indeed, epitope hierarchy and immunodominance may be determined by proteasomal cleavage preferences [16–18].

[I 19055]

Abbreviations: **ER:** Endoplasmic reticulum **TAP:** Transporter associated with antigen presentation **NP:** Nucleoprotein **HA:** Hemagglutinin

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Several post-translational protein modifications were shown to be able to target cytosolic proteins for rapid degradation. Rapid proteolytic turnover of many intracellular proteins requires the covalent attachment of ubiquitin to a lysine side chain(s) of the substrate. Ubiquitination of target proteins is mediated by several ubiquitin ligases (called E3, reviewed in [19]) that can recognize motifs within the protein or modifications of the protein, such as phosphorylation, and assist in the covalent binding of the ubiquitin moiety. The role of ubiquitination of target proteins for the generation of CTL epitopes remains controversial [20, 21].

Post-translational protein modification may also have a more direct impact on antigen presentation [22–24]. Neefjes and colleagues [3] demonstrated that peptides with a blocked N terminus failed to be efficiently translocated by the TAP complex into the lumen of the ER. It was also shown that methylation or acetylation of the N terminus of a 9-mer peptide reduces the binding affinity between the peptide and MHC class I molecules [25, 26]. This raises the possibility that presentation of N-terminally located epitopes might be affected by N-alpha acetylation found in the majority of cellular proteins [27]. We therefore sought to address whether the position of CTL epitopes within newly synthesized proteins may affect their presentation on the cell surface in association with MHC class I molecules. An earlier report showed that generation of an L^d epitope from the IE1 protein pp89 (a regulatory protein of murine CMV) was impaired by its proximity to the protein N-terminus [16]. We decided therefore to examine the presentation of epitopes which are in close proximity to the N terminus and the possibility that a modification may affect their presentation.

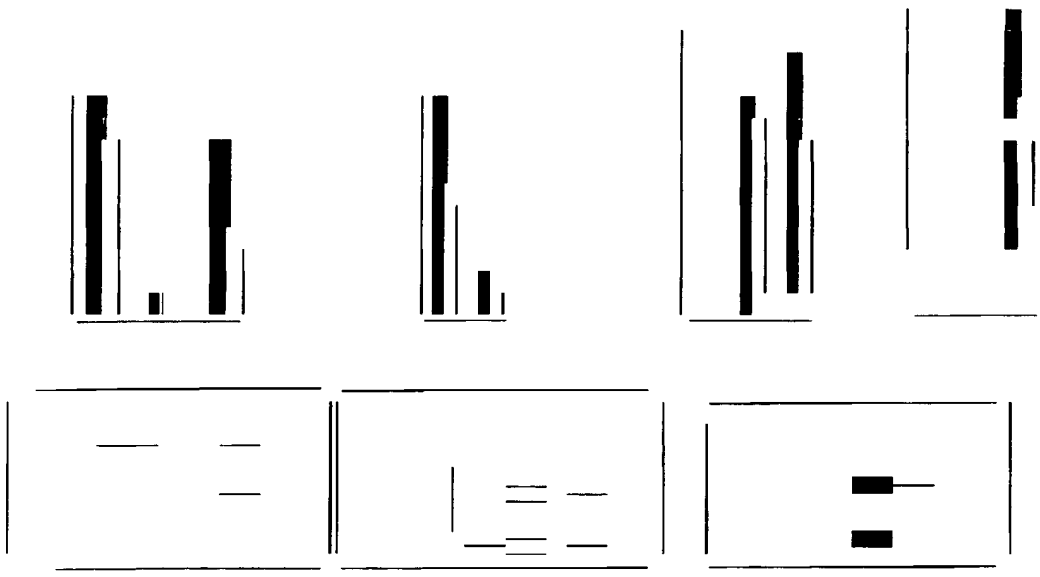
2 Results

2.1 Lack of presentation of N-terminal CTL epitopes

We sought to study whether close proximity of CTL epitopes to N terminus of target proteins may influence their efficiency of presentation through MHC class I molecules. We engineered a recombinant vaccinia virus (M-) expressing the D^b epitope 366-374 at the N terminus of a fragment of the influenza nucleoprotein (NP) (the nomenclature of the vaccinia constructs used is summarized in Table 1). As a control we used recombinant vaccinia virus expressing either the 9-mer peptide 366-374 (Pep vac) or a longer NP fragment, containing the 366-374 epitope several amino acids downstream of the N-terminal methionine (IMP). We demonstrated that presentation of the D^b 366-374 epitope was abolished in target cells infected by M-vaccinia, while efficient presentation was observed after infection with the control vaccinia viruses (Fig. 1A). The block in presentation after infection with M-vaccinia was specific for the N-terminal 366-374 D^b epitope, since the downstream epitope (NP 380-388) was efficiently presented through HLA B8 molecules (data not shown). To further characterize the block in presentation of N-terminal epitopes and to study whether such a block in presentation could be detected with other N-terminal epitopes, we analyzed presentation of the NP epitope 147-155 restricted through K^d (M-K^d). We also extended this analysis to the presentation of a CTL epitope naturally occurring at the N terminus of a cytosolic protein, the melanoma BAGE epitope 2-10, presented through the HLA Cw16 molecules [28]. Recombinant vaccinia viruses were engineered encoding either epitopes positioned at the N terminus of the NP fragment 375-498 (Fig. 1B and C). As a control for presentation of the 147-155 NP epitope, we used a recombinant vaccinia virus encoding an NP fragment containing the 147-155 epitope several amino acids downstream of the N terminus (IMP-K^d). As a positive control for presentation of the 2-10 BAGE epitope, a vaccinia encoding the full-length BAGE protein was used. We demonstrated that the presentation of the NP CTL epitope 147-155 was abolished by its location at the N terminus of the NP fragment 375-498 (Fig. 1B), while the BAGE 2-10 epitope was efficiently presented (Fig. 1C). Presentation of the N terminal, BAGE Cw16

Table 1. Nomenclature of the constructs

Abbreviation	Details of constructs expressed by the vaccinia
M-	Met 366-498 of NP
MA	Met Ala 366-498 of NP
MV	Met Val 366-498 of NP
MG	Met Gly 366-498 of NP
MD	Met Asp 366-498 of NP
ME	Met Glu 366-498 of NP
MA_2	Met Ala Ala 366-498 of NP
MA_5	Met Ala Ala Ala Ala Ala 366-498 of NP
IMP	M327-498 of NP
IMP-K^d	Met 327-365,147-155,375-498 of NP
L+M-	Hemagglutinin leader +Met 366-498 of NP
MK^d	Met 147-155,375-498 of NP
BAGE	1-43 of BAGE
BAGE (NP)	1-10 of BAGE 375-498 of NP
MK^dD^b	Met 147-155,366-498 of NP
D^b/21 mer	Met 366-386 of NP
D^b/43 mer	Met 366-408 of NP
Pep vac	Met 366-374 of NP



presentation of N-terminal epitopes can be extended to a second CTL epitope but it is not universal. They suggest that epitopes primary sequences may be important in determining whether close proximity to the protein's N terminus affects their presentation to CTL.

2.2 N terminal block in antigen presentation is a cytosolic phenomenon controlled by events occurring at the N terminus of the polypeptide target protein

To identify the cellular compartment controlling the block in presentation of the N terminal 366-374 epitope, we engineered a recombinant vaccinia virus expressing the NP fragment 366-498 preceded by the influenza hemagglutinin (HA) leader sequence (L+M- in Fig. 2). The presence of the HA leader sequence ensured the targeting of the NP fragment into the ER lumen [29]. Our results demonstrate that the block in presentation of the 366-374 N-terminal epitope was a cytosolic phenomenon, since it could be relieved be translocating the NP fragment into the ER lumen for lysis by 366-374-specific CTL (Fig. 2B). This confirmed that the 366-374 epitope, preceded by the HA leader sequence, was processed in the lumen of the ER. As a control, we showed that transfection of .174/Db cells with TAP1/2 cDNA failed to relieve the block in presentation of the cytosolic N-terminal 366-374 D^b epitope (Fig. 2C).

We then sought to address whether events a the N terminus of the target protein were responsible for decreasing the efficiency of presentation of the NP366-374 epitope. We approached this issue by following two distinct strategies. The first was to distance the epitope from the N terminus by an increasing number of amino acid residues (Fig. 3A). The second was to mutate the epitope flanking residues, by introducing different N terminal residues, and compare their ability to overcome the N terminal presentation block (Fig. 3B). We demonstrated that separating the 366-374 epitope from the N terminus by two residues was sufficient to relieve the antigen presentation block, while a single alanine residue had no effect (Fig. 3A). To analyze whether epitope N terminal

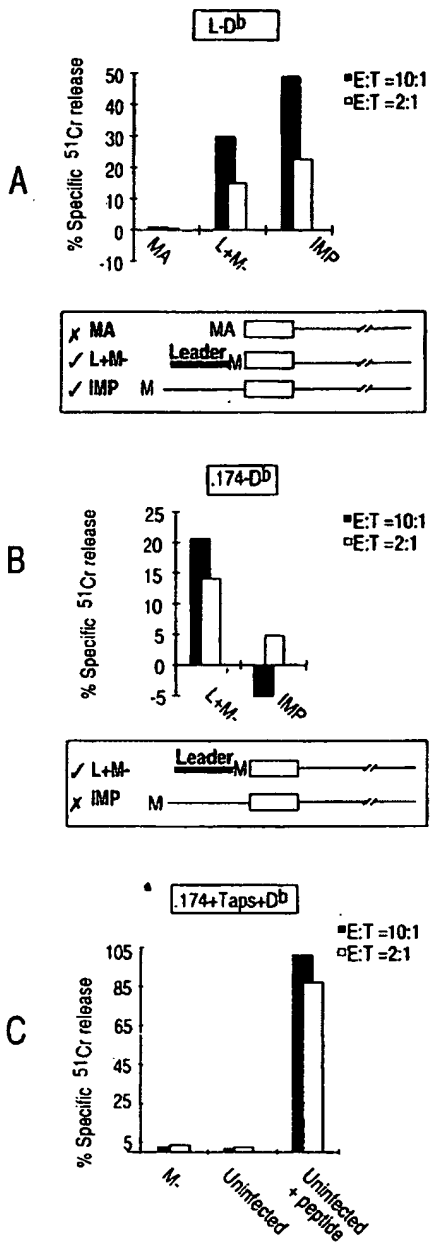


Figure 2. The N-terminal block of antigen presentation is a cytosolic phenomenon. Target cells and recombinant vaccinia viruses are shown in boxed panels. Presentation of D^b epitope NP 366-374 was analyzed in all the panels. Killing or lack of killing of target cells is summarized in boxed panels by the symbols ✓ and ✗, respectively.

flanking residues may relieve the antigen presentation block, we introduced different N-terminal residues upstream of the 366-374 epitope. In Fig. 3A we show that the presence of a single alanine failed to relieve the antigen presentation block. Similar results were obtained after introducing negatively charged residues such as glutamic acid or aspartic acid (*i.e.* ME and MD) in the same upstream position (Fig. 3B). In contrast, other residues such as valine or glycine (*i.e.* MV and MG) were capable of relieving the block in presentation of the 366-374 epitope. These results define two points: firstly, that the distance from the N terminus is of importance in controlling the N terminal block in presentation, and secondly that the N terminal block in presentation is controlled by the epitope flanking residues. These results strongly suggest that events occurring in close proximity of the epitope are responsible for its lack of presentation. Furthermore, we showed that shortening the NP fragment from the C terminus to an either 21 or 43 amino acid long fragment (*i.e.* D^b/21-mer and D^b/43-mer) failed to relieve the block in presentation (Fig. 3C), suggesting that events that cause the presentation block are not mediated by sequences downstream of the first 21 amino acid residues.

2.3 Presentation of the N-terminal epitope in different mutants correlates with a difference in electrophoretic migration of the antigenic proteins

We ruled out that the effect of the different epitope flanking residues in the presentation of the N-terminal epitope 366-347 was due to an effect on the overall half life of the target proteins, since two constructs with different presentation phenotypes (MA and MV) differed only slightly in their half lives (data not shown). However, we were surprised to observe that the migration of the MA and MV proteins on SDS-PAGE (Fig. 4A) was slightly different. To rule out an intrinsic variability of different constructs in their mobility on-SDS-PAGE, we extended this analysis to other vaccinia NP constructs. The results showed that all the fragments which were unable to sensitize target cells for lysis by 366-374-specific CTL (*i.e.* M-, MA, MD and ME) had a similar electrophoretic mobility, which differed from the mobility of the MV and MG fragments (Fig. 4A and B). The results of this analysis revealed a correlation between the migration pattern of the different constructs on SDS-PAGE gels and their ability to present the D^b epitope. Constructs which did not sensitize target cells for lysis through D^b molecules migrated slower than constructs which were efficiently presented (Fig. 4A). It is of particular interest that the ER-resident 366-498 construct (L+M-), which sensitizes target cells for lysis, has a faster mobility on SDS-PAGE than cytosolic fragments which failed to be seen by 366-374-specific CTL (MA) (Fig. 4C). As a control for the shift in size of the MA and MV constructs, we also analyzed constructs containing two and five alanine residues, downstream of the N-terminal methionine (Fig. 4C).

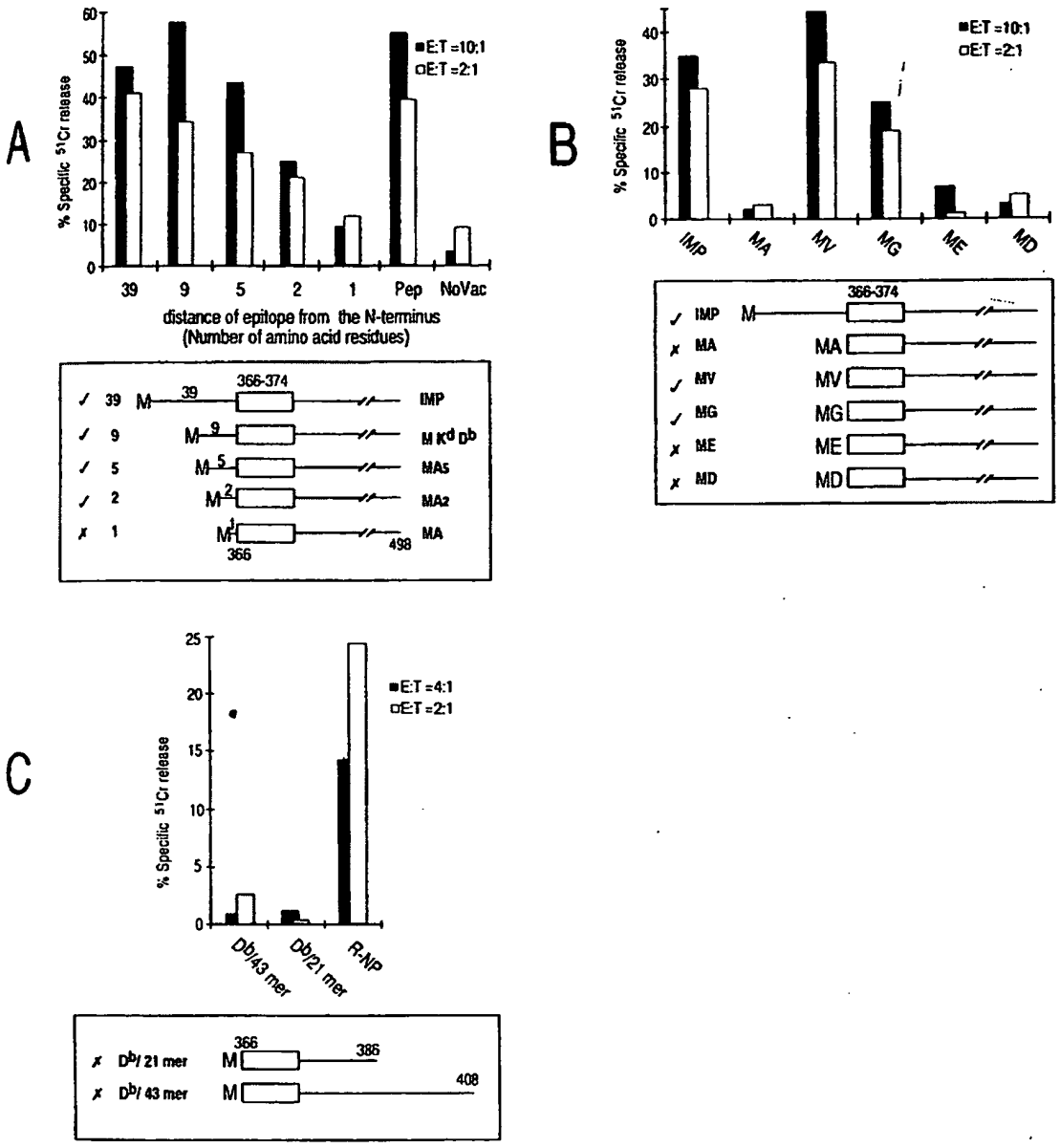


Figure 3. The effect of epitope flanking sequences on the N-terminal block of antigen presentation. LD^b cells were infected with recombinant vaccinia viruses shown in boxed panels. Presentation of the D^b epitope NP 366-374 was analyzed in all the panels. Killing or lack of killing of target cells is summarized in boxed panels by the symbols ✓ and ✗, respectively.

A confirmation was sought for the correlation between the change in the proteins' SDS-PAGE mobility and their ability to present the D^b epitope by separating the different constructs on a denaturing IEF-PAGE and comparing their isoelectric points (pI). (Fig. 4C). Results of these experiments confirmed that cytosolic (MV) and ER resident (L+M-) NP fragments, which were both capable of sensitizing target cells for lysis by 366-374-specific CTL, had a similar pI. In contrast, the MA NP fragment, whose primary sequence contains the same charged residues as the L+M- and MV fragments, had a different pI. The difference in pI between the MA NP fragment and MV NP fragment was consistent with either the addition of a negatively charged residue or the absence of a positively charged residue. These results reinforced the correlation between the biochemical change in protein and the antigen presentation block.

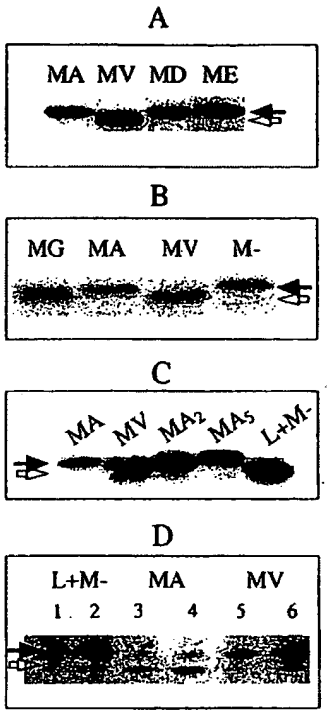


Figure 4. Difference in electrophoretic mobility of the truncated NP variants. Anti-NP antibodies were used to immunoprecipitate vaccinia-encoded NP mutants from metabolically labeled LD^b cells. Immunoprecipitated products were separated by either SDS-PAGE (**A, B, C**) or IEF-PAGE (**D**). In (**D**), cells were metabolically labeled either for 30 min (lanes 1, 3 and 5) or 6 h (lanes 2, 4 and 6). Outlined arrows indicate constructs with a faster SDS-PAGE mobility (**A, B** and **C**) and lower pI (**D**) as compared to the other constructs (black arrows).

2.4 Lack of presentation of N-terminal CTL epitopes *in vivo*

We decided to examine the block in presentation of the N-terminal epitope *in vivo*. Two constructs were compared, one in which presentation of the N-terminal epitope was blocked (MA), and the other in which the epitope was presented (MV). C57BL/6 mice were primed with influenza virus and subsequently challenged with either MV or MA. Vaccinia virus titers were determined by counting PFU in the ovaries, 5 days after infection. At this time point vaccinia viruses, capable of sensitizing infected cells for lysis by 366-374 epitope-specific CTL, were expected to be cleared by the primed mice. As controls, the MV or MA vaccinia viruses were injected into mice that were not primed with influenza virus and were thus expected to produce high titers of both vaccinia virus. Fig. 5 shows that in primed mice, only the vaccinia containing the MA construct could proliferate,

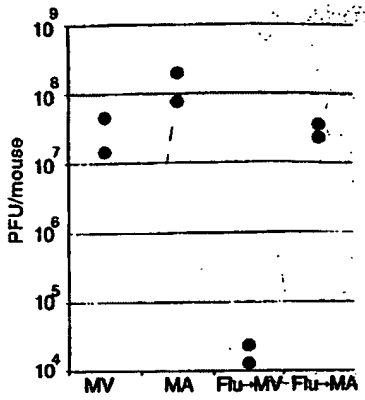


Figure 5. The N-terminal block of antigen presentation prevents clearance of an acute viral infection in mice. Mice primed with influenza virus were challenged with recombinant vaccinia virus encoding either MA-NP fragment or MV-NP fragment. Virus titer in the ovaries was determined (see Sect. 4.7). Each circle represents a single mouse and shows the number of PFU per two ovaries. MA and MV are control mice challenged with either MA or MV vaccinia virus but not primed with influenza virus. Flu → MA and Flu → MV are mice primed with influenza virus and challenged on day 20 with vaccinia virus containing either MA-NP fragment (Flu→MA) or MV-NP fragment (Flu→MV).

demonstrating the significance *in vivo* of the N-terminal block in presentation of the 366-374 epitope.

3 Discussion

We have identified a block in the antigen presentation pathway which is dependent on the epitope's position within the protein. This presentation block reduces the ability of primed mice to clear acute viral infection. We have located the cellular compartment responsible for this block in presentation and demonstrated that it can be relieved by distancing the epitope from the N terminus or by changing the immediate upstream residues. This phenomenon is not specific for one epitope, as it was reproduced with a second epitope presented through H2-K^d class I molecules. We showed, however, that the N-terminal block in antigen presentation is not universal, as evident from presentation of the N-terminal Cw16 BAGE epitope. We went on to demonstrate that the block in presentation of N-terminal epitopes correlates with a shift in size and charge of the target protein. Our results show a significant correlation between the lack of presentation of N-terminal epitopes and mobility on SDS-PAGE and pI of vaccinia-encoded NP isolates. Fragments which are not presented to NP366-374-specific CTL have a slower mobility on SDS-PAGE gels and also have a more acidic pI. The nature of the modifi-

the coding sequence of M- (generating an additional methionine codon between them). The BAGE 1 gene was amplified from cDNA clone [28] using primers GAAGATCTCAGAAGATGAAGCACAGAGC and GAAGATCTCAGAAGATGAAGCACAGAGC and inserted between the NcoI and BglII sites of pSC1130R.2. The nomenclature of the constructs is summarized in Table 1.

4.3 CTL lines and cytotoxicity

Isolation and maintenance of CTL clone F5 recognizing the H-2D^b-restricted epitope (ASNENMDAM) of influenza virus (A/HK/8/68) NP were described previously [32]. Isolation of clone 82/82 recognizing the Cw*1601-restricted epitope of the BAGE protein was described elsewhere [28].

4.4 Chromium-release assay

CTL assays were performed using a standard protocol. Target cells (1×10^6) were infected with 10^7 PFU of recombinant vaccinia virus and labeled with 100 μCi ^{51}Cr at 37 °C. After 90 min, cells were washed twice and resuspended in R10 medium for 4 h at 37 °C. Targets were added to CTL, R10 only, or 5 % Triton. After 4 or 7 h incubation at 37 °C, supernatants were collected and counted. Specific lysis was calculated as mean of duplicate results of release with added CTL minus release without CTL (in R10) divided by release in 5 % Triton minus release in R10.

4.5 Immunoprecipitation

Cells were infected with recombination vaccinia virus at a multiplicity of infection of 10 for 90 min at 37 °C, then incubated for 2 h in R10 following a wash in the same medium. Cells were then resuspended at 2×10^7/ml in methionine- and cysteine-free R10 for 1 h at 37 °C. Promix (143 μCi; 70 % [^{35}S]methionine, 30% [^{35}S]cysteine) was then added and the mixture incubated for 30 min (unless stated otherwise) at 37 °C. Labeled cells were then washed in ice-cold PBS and resuspended in 0.5 ml lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.5 % NP40, 0.5 % Mega9, 2 mM phenylmethylsulfonyl fluoride, 5 mM Iodoacetate), followed by centrifugation to pellet nuclei. Lysates were precleared with Pansorbin overnight at 4 °C and immunoprecipitated with sheep antiserum (S146) raised against NP (kindly provided by Prof. RW Newman, Potters Bar, GB) and protein-A-coated Sepharose beads. Following extensive washing, proteins were eluted from the beads using either standard SDS-PAGE sample buffer and heated at 95 °C for 5 min or in IEF-sample buffer (9.5 M urea, 2 % NP40, 2 % ampholines pH 3.5-9.5, 5 % 2-ME) and incubated at room temperature for 60 min.

4.6 IEF and SDS-PAGE

For IEF gels, eluted proteins were separated by denaturing IEF-PAGE containing 3.85 % acrylamide, 7.8M urea, 1.7 % NP40, and 3.4 % and 0.85 % ampholines pH 3.5-5 and 3.5-9.9, respectively. Samples were loaded at the high pH end of the gel and a constant voltage of 880V was applied overnight (13-16 h). For SDS-PAGE, proteins were separated on a 12 % acrylamide gel. SDS-PAGE gels were stained with Coomassie Blue. Gels were then soaked in Amplify (Amersham), dried and exposed to X-ray film.

4.7 Infection of mice

Four female C57BL/6 mice were primed by intranasal infection with influenza virus E61-13-H17 [35]. Four control female mice were left uninfected. At day 20 two primed mice and two control mice were infected i.p., with recombinant vaccinia virus expressing either the MV construct or the MA constructs. Five days later, ovaries were collected and the number of vaccinia PFU was determined by infecting dilutions of homogenized ovaries onto TK143 cells and counting plaques after 2 days.

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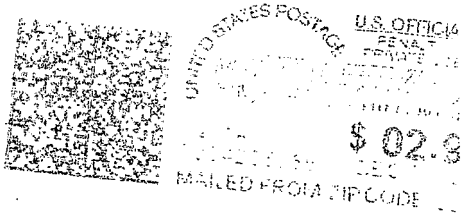

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